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Carcinogenic Action of Refined Tumor Factor Isolated from Mouse Leukemia Tissue.* (24883)

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Induction of leukemia and solid neoplasms in susceptible mice by injection of crude, cell-free filtrates of leukemic tissues has been reviewed by Gross(1). These crude, cell-free filtrates are heterogeneous, and contain a small amount of labile carcinogenic material along with enormous quantity of impurities (2). We had previously postulated(3) that variability in activity, lability, lack of reproducibility, etc., of crude filtrates could be eliminated by refining them. First attempts to purify the tumor factor by standard viral purification technics such as cold methanol fractionation(4), protamine precipitation(5) and genitron homogenization(6) were not successful. This communication presents results obtained when a modification of the method developed by Burton *et al.*(7) for purification of tumor-inducing factor (TIF) of *Drosophila* was employed.

Methods. Strains of mice used were originally obtained from Dr. Ludwik Gross and inbred in our lab for 3½ years. Incidence of spontaneous leukemia in AK (donor) strain has been approximately 85%, whereas only one spontaneous leukemia was found in several thousands of our C3H(f) host strain. We have not observed other spontaneous neoplasms in our C3H(f) line. Since, unlike the AK-C3H(f) hybrids, this

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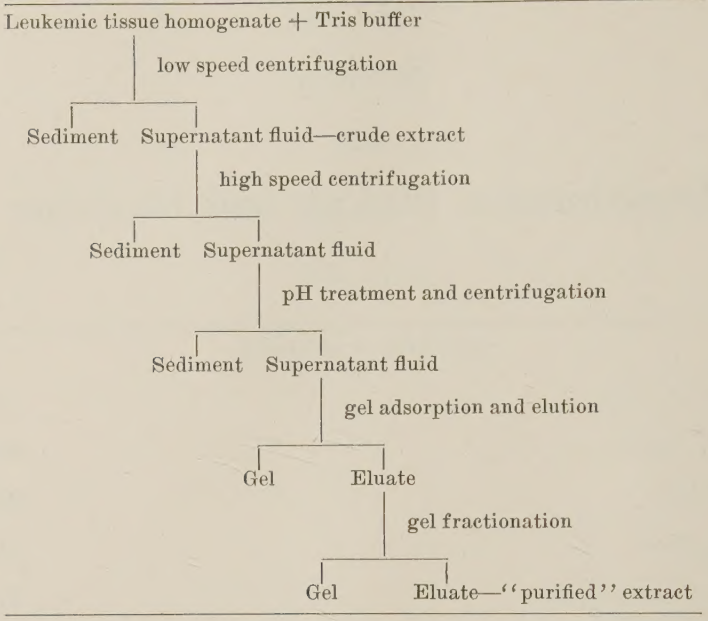
TABLE I. Modified Purification Process.
(After Burton *et al.*)



C3H(f) line apparently has a high resistance to spontaneous neoplasia, it was selected as source of host animals. Purification methods of Burton *et al.*(7) were modified where laboratory facilities and differences in tissue made it necessary. An outline of refinement process is presented in Table I. Heat treatment, ammonium sulfate precipitation and dialysis used by Burton *et al.*(7) in earlier work were omitted. Tissues (liver, spleen, lymph nodes, thymus, brain) from AK mice with spontaneous or transplanted leukemia and from C3H(f) mice with induced leukemia were homogenized in "tris" buffer, differentially centrifuged, pH treated, adsorbed on calcium phosphate gel, and eluted; these refined extracts were then ampulated and stored in dry ice. When 3 to 48 hours old, C3H(f) mice were inoculated either subcutaneously with 0.1 ml, or intravenously with 0.03 - 0.05 ml, of the extract. At weaning, surviving animals were toe clipped for identification and caged in groups of 8 or 10. Water and Purina chow were provided *ad lib.* and the diet supplemented with whole oats twice a week.

Results. The results clearly indicate high degree of tumor-inducing activity of extracts prepared by this technic (Table II: A, B, C and E). This activity was reproducible in 2 extracts prepared from different tissue sources at different times, and total tumor incidence appeared more dependent upon dosage than upon route of administration (Table II: Compare A with B and E). In each experiment where tumors were found, they were first palpable from 75 to 100 days post inoculation, but were suspected as early as 50 days in many animals. Induced fibrosarcomas and the parotid, adrenal and mammary carcinomas were histologically comparable to previous descriptions of such tumors(8,9,10).

The buffer was negative for tumor-inducing activity, confirmed by microscopic examination of histological sections of parotid glands from experimental animals, (Table II: F). Negative results from material stored at 0°C (Table II: G) showed lability of tumor factor preparations and the need for storage in dry ice, and necessity for adhering strictly to preparative procedure was shown by negative results when high speed centrifugation was omitted.

Discussion. A procedure has been developed for extracting and refining tumor factor (TF) contained in mouse leukemic tissue. The refined preparation repeatedly induced a

TABLE II. Tumor Induction with Purified Extract.

Exp.	Survived to weaning of No. inj.	Extract and No. days stored before use	Dose (ml) and route	No. tumors and tumor animals	Sacrificed at avg age of (days)	Tumor type and No.
A	9/26	#20, 3-5 days in dry ice	.1 SC	17/9	136	10 PT 1 FSa 4 MT 1 Myx 1 Adr
B	30/38	#20, 112 days in dry ice	.03–.05 IV	38/20	167	31 PT 3 MT 4 FSa
C	9/25	#20, 150 days in dry ice	.1 SC	20/9	147	16 PT 3 MT 1 OSa
D	"	#20, 30 days at 0°C	*Idem*	0	210	0
E	37/66	#1	.01–.05 SC	33/22	140	31 PT 1 MT 1 FSa
F	27/44	Tris 0.05 M pH 7.5 controls	.05 IV	0	240	0
	16/16		.1 SC	0	210 Still alive, no gross evidence of tumor	0
G	155/333	Prep. modified by omitting high speed centrifugation.	.05 IV .1 SC	0	210	0

PT, parotid gland carcinoma; MT, mammary carcinoma; FSa, fibrosarcoma; Myx, myxoma; Adr, adrenal ca.; OSa, osteogenic sarcoma.

Total 108 tumors

60 animals

variety of neoplasms in a high percentage of inoculated animals. Reproducibility of TF activity in repeated experiments involving large numbers of hosts from different litters, and predictable time of tumor appearance, testify to the enhanced potency achieved by using refined extracts of tumor tissue. Mammary tumors develop at an even earlier age than is reported when "milk factor" is injected. At 76 days it was suspected in one male animal, and when this animal was sacrificed at 104 days bilateral parotid tumors were found as well as mammary tumor. This accelerated tumor development gives to the method a decided advantage over use of crude-cell-free filtrates, and also the advantage of being subject to more precise control than tissue culture method.

That no leukemia developed in the experimental animals, but only a variety of solid tumors, is subject to several interpretations: as the action of a family of tumor viruses(1); as the action of one virus, *i.e.*, that called "polyoma" by Stewart and Eddy(10) and "pleuripotent" by Furth *et al.*(11); as the action of a single tumor factor in a preparation containing substances which modify the action of this factor. Different titers or ratios of these biologically active modifying substances, which may be concentrated or lost during the purification process, may govern the induction of specific tumors by TF.

Masking of tumor factor present in tissue extracts and the possibility that purification technics might overcome such masking has been discussed previously in a review of Hodgkin's disease(3). It was suggested then that the Drosophila technics of Burton and Friedman might be successfully applied to mammalian tissues.

That "tumor factor" could be isolated from sources so different as Drosophila and mice by use of similar technics has suggested the attempt to find this factor in Drosophila, fish lymphoma, chicken lymphoma, Lucké frog kidney tumor, etc. Should factors isolated

from such diverse sources give rise to the same variety of tumors in the C3H(f) mouse as have been found in the experiment just completed, a tumor-inducing mechanism common to Metazoan life will have been demonstrated for the first time.

The successful application of purification technics to the isolation of tumor factor from Hodgkin's disease tissue and other neoplastic tissues from human beings will be presented later.

Summary. 1) A procedure has been developed for extracting and refining tumor factor contained in mouse leukemic tissue. The refined preparation repeatedly induced a variety of neoplasms in high percentage of inoculated animals, but no leukemia was induced. 2) Results were reproducible and time of tumor appearance predictable. Tumors developed at earlier age than when crude cell-free filtrates are used. 3) This work suggests that an attempt be made to isolate tumor factor from Drosophila, fish lymphoma, chicken lymphoma, Lucké frog kidney tumor, etc., so that a tumor-inducing mechanism common to Metazoan life may be demonstrable. 4) By purification technic discussed, tumor factor has been isolated from Hodgkin's disease tissue and other neoplastic tissues from human beings to be reported.

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Immunization Against Ehrlich's Ascites Carcinoma with X-Irradiated Tumor Cells.* (24884)

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In 1910 Contamin(1) produced immunity to transplanted tumors in mice by pre-treatment with tumor cells that had been x-irradiated *in vitro*. Since that time varying degrees of immunity have been obtained against mouse sarcoma-180(2) and mouse Ehrlich ascites tumor(3) following treatment with x-irradiated cells. In the present study, immunization procedures with irradiated cells were carried out both before and after challenge with Ehrlich's ascites carcinoma.

Methods. Swiss mice of mixed sexes were used. They weighed from 17 to 20 g when experiments were initiated. The material used for immunization and challenge injections consisted of pooled ascites exudate collected from 2 or more mice that had received intraperitoneal (ip) transplants one to 3 weeks previously. Five units of heparin were added to each ml of exudate when ascites fluid was collected to prevent clotting. Tumor cells employed for challenge transplants were withdrawn from the peritoneal cavity, counted with a hemacytometer, diluted with physiological saline solution (PSS) and injected into recipient mice within one hour from time of collection. The number of tumor cells used in the challenge injection varied in different experiments. Tumor cells used for immuniza-

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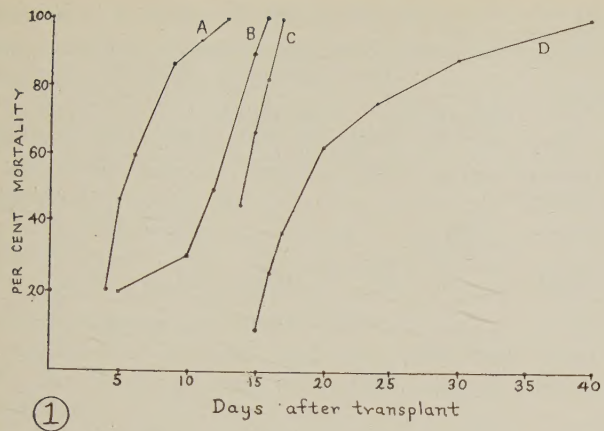

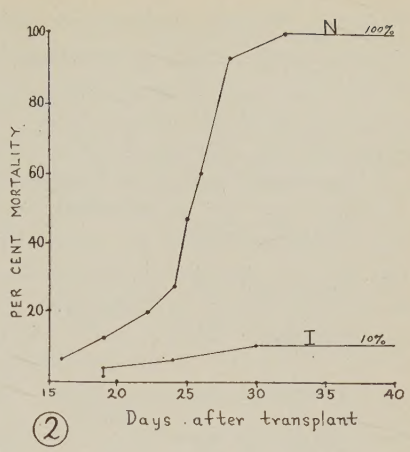

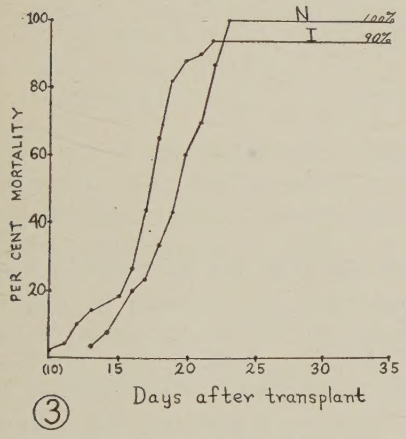

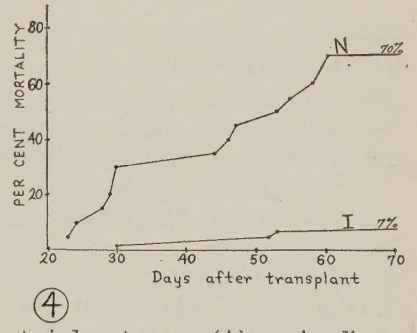

FIG. 1. Effect of different irradiation doses on capacity to induce tumors. (A) non-irradiated cells, (B) cells exposed to 500 r, (C) 1000 r, (D) 2500 r.

FIG. 2. Immunization with single inj. of irradiated tumor cells prior to ip challenge. (I) Immunized mice, (N) non-immunized controls.

FIG. 3. Immunization with irradiated cells after ip challenge. (I) Immunized mice, (N) non-immunized controls.

FIG. 4. Immunization with irradiated cells after sc challenge. (I) Immunized mice, (N) non-immunized controls.

tion were prepared as follows: 20 ml of heparinized ascites fluid were placed in a plastic petri dish, exposed to x-irradiation in air, and diluted wth PSS solution to give the desired cellular concentration. The x-ray factors used were: 250 K. V., 15 ma, hvl 0.77 mm cu, t.o.d. 40 cm, output 280 r/min. In all cases immunizing injections were administered within an hour after x-ray exposure. Irradiation dose and number of cells in the vaccine varied. When mice received multiple injections, fresh vaccines were prepared each day that immunizing injections were administered.

Results. To determine what dose of x-irradiation was necessary to interfere with successful tumor transplant, the ascites fluid was exposed to varying doses (500 to 100,000 r) of x-irradiation *in vitro*. Mice were divided into groups of 10 and given single ip injections containing 5,000,000 cells that had received different irradiation exposures. Twenty mice served as controls and received non-irradiated cells. Daily mortalities following these injections are recorded in Fig. 1. In no case did tumors develop if the irradiation dose was 4,000 r or greater. Relationships between mortality curves in Fig. 1 are

TABLE I. Effective Immunization against Ehrlich's Ascites Carcinoma with Cells Exposed to X-irradiation *In Vitro*.

			Immunization				Tumor transplant		Mortality		
Exp.	Group	No. mice	Start*	Inj.†	X-ray	Cells/inj. × 1000	Route	No. cells × 1000	Ratio	%	P value‡
1	Immunized	50	20 days before transplant	1	4,000 to 13,000 r	5,000	IP	2	5/50	10	<.001
	Control	15		0		Non-immunized	"	"	15/15	100	
2	Immunized	50	1 day after transplant	5§	10,000 r	20	"	16.5	45/50	90	>.1
	Control	30		0		Non-immunized	"	"	30/30	100	
3	Immunized	55	1 day after transplant	5§	10,000 r	20	SC	"	4/55	7	<.001
	Control	20		0		Non-immunized	"	"	14/20	70	

* Time that single or first inj. of irradiated cells was given in relation to inj. of viable cells.
† All immunizing inj. were given by intraper. route.
‡ P values calculated by Chi square.
§ The 5 immunizing inj. were administered over a 10-day period.

similar to those seen when mice are challenged with different numbers of cells. Consequently, increase in survival time that follows an increase in irradiation dose might be due to a decrease in number of viable cells.

In the first immunization experiment, irradiated cells were administered prior to challenge with viable non-irradiated cells. The 50 mice in the immunized group received an ip injection of 5,000,000 cells that had been irradiated *in vitro* with doses from 4,000 to 13,000 r. Twenty days after the single injection of irradiated cells the immunized mice and a control group of normal mice received ip transplants containing 2,000 non-irradiated cells/mouse. Mortality curves (Fig. 2), demonstrate the protective effect of immunization on subsequent challenge. Other pre-immunization experiments have been carried out and essentially the same results obtained.

An attempt was made to protect mice against the tumor by initiating immunization procedures after tumor transplantation. The mice were challenged by either ip or subcutaneous route with 16,500 nonirradiated cells. Both routes of challenge were employed to see if different results would be seen between the rapidly growing tumor in the peritoneal cavity and the slower growing tumor in subcutaneous tissues. One day after challenge the first immunization injection was given. The immunization schedule consisted of 5 ip injections over a 10-day period. Each immunizing injection contained 20,000 cells that had received a 10,000 r x-ray exposure. Daily mortalities following the ip challenge and post-immunization are recorded in Fig. 3. Even though mice in the immunized group seemed to die faster, 10% survived; whereas, all control animals died. The difference in mortality between immunized and normal mice is more pronounced following the sc challenge (Fig. 4). A 10-fold reduction in mortality was obtained when immunization was started following sc transplantations. These results have been confirmed in recent experiments. Results of immunization experiments are summarized in Table I.

In addition to the challenge experiments in which mortality was measured, attempts were made to determine if Ehrlich's ascites cells could be isolated from immunized mice that had received ip transplants but did not develop tumors. Thirty-three to 40 days after ip challenge, the survivors were sacrificed and their peritoneal cavities washed with 5 ml of PSS. Two and one-half ml of this PSS containing the peritoneal cells was then injected into normal mice. Of 60 mice injected with these exudates, only one developed an Ehrlich's ascites tumor. Since we have been able to induce tumors with as few as 2 normal tu-

mor cells, it is speculated that immunization has a lethal rather than a static effect on transplanted tumors.

Discussion. McKee *et al.*(3) were unable to induce immunity by treatment with Ehrlich's ascites cells that had been killed by desiccation, freezing and thawing, mechanical grinding, and supersonic waves. The possibility exists that the physical-chemical changes of tumor antigens induced by *in vitro* x-irradiation are extensive enough to make the antigens less homologous and more antigenic for the mouse without destroying antigenic specificity. Indirect evidence for this is manifested by recent observations made in this laboratory. The agglutinins against normal Ehrlich's tumor cells are more readily produced and demonstrated in sera of animals that have been exposed to higher irradiation doses.

Summary. Pre-immunization with irradiated Ehrlich's ascites tumor cells protected mice against subsequent ip transplants of viable cells. Immunization procedures started one day after sc challenge with the tumor, significantly reduced mortality. Such post-immunization did not inhibit the rapid multiplication that follows ip transplantation of the tumor. The evidence indicated that the immunization procedures employed resulted in a lethal rather than a static effect on transplanted tumor cells.

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Passage of Bacteriophages from Mother to Foetus in the Rat.* (24885)

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During a study of tolerance to homografts produced in rats by direct injection of particulate antigens into foetuses, the question arose whether similar antigens can cross the intact placental barrier under natural conditions. Passage of antibodies and similar large molecules from mother to foetus has been shown by studies of Brambell and his associates(1). Whether larger particles can cross the placental barrier has not been answered satisfactorily. Bacteriophages offer special advantages in such a study. They are larger than protein molecules, they multiply only in specific host bacteria and their presence can easily be detected by testing for plaque forming activity, produced only by intact viable phage. Since phage is not able to penetrate and multiply in cells other than susceptible bacteria, it is free from the objection that may be raised against animal viruses, namely, that the latter may traverse the placenta by direct extension of an infectious process. The passage of coliphage and staphylococcal phage across the placental barrier was investigated by Grasset(2), who obtained no evidence for such passage in rabbits and guinea pigs; Blair and Reeves(3), using high titer phage suspensions, reported passage in the guinea pig. However, Nattan-Larrier *et al.*(4) were unable to repeat the latter results. Burckhardt(5) reported passage of coliphage from mother to foetal blood in guinea pigs. Recently Keller and Engley(6) and Keller(7) published quantitative studies on passage of *Bacillus megatherium phage* through gastrointestinal and renal barriers and through intact skin of mice.

Materials and methods. Four bacterio-

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phages of different sizes were employed. These included coliphages T3 (head diameter 47 mμ, tail length 15 mμ) and T1 (head diameter 50 mμ, tail length 150 mμ)(8); mycobacteriophages D29 (head diameter 65 mμ, tail length 125 mμ) and D32 (head diameter 80 mμ, tail length 220 mμ)(9). High titer phage suspensions, 10^8 to 10^{10} particles/ml, were prepared as described by Adams(10) for coliphage and by Sellers *et al.*(9) for mycobacteriophage. The preparations were either non-filtered lysates or lysates which had been passed through ultra-fine sintered glass filters. In addition, a few experiments were performed with phage suspensions which had been washed and purified by repeated cycles of high speed centrifugation. Long-Evans rats at different stages in second half of gestation were employed. Phage suspensions were injected by intravenous or intrauterine route, except for 2 instances of intracardiac injection. Intrauterine injections were made after opening abdomen as described by Brambell *et al.*(1). The mother was anesthetized with ether at various intervals after injection, and foetuses removed with foetal membranes intact. The membranes were washed in saline followed by 70% alcohol and dried with sterile gauze before foetal fluids were collected with sterile, finely drawn, capillary pipettes. The foetuses were then freed of all membranes and dipped in 70% alcohol or 5% Chlorox solution to free the outside of body from any contamination with phage from maternal blood. The chest of the foetus was opened aseptically and blood obtained by cardiac puncture with sterile capillary pipettes. Since the quantity of blood from each foetus was small, the majority of samples were pooled, each sample consisting of blood from 3 or 4 embryos. When foetuses were too small (10-12 days old) for bleeding, whole foetuses were ground in sterile glass homogeniser and tested for phage. In some instances, blood, peritoneal fluid, and spleen from the mother were also tested. In assaying for phage, samples (blood, foetal fluids, homogenate, etc.) were placed in flasks containing approximately 20 ml of heart infusion broth. Five ml of actively growing (log phase) culture of susceptible bacteria were then added and the flasks incubated, 4 hours for coliphage and overnight for mycobacteriophage. Presence of phage in broth culture was detected by spotting aliquots on soft agar Petri plates seeded with susceptible bacteria(10). Typical plaques indicate phage activity.

Results. Twenty-three pregnant rats were injected with coliphage preparations and foetuses sampled to 24 hours later. Phage T3, smallest of the T phages, was employed in first experiments and results with T3 lysate are recorded in Exp. 1, Table I. Samples of mothers' blood, peritoneal fluid, and spleen contained phage. That phage remained viable in the animal (spleen) to 23 hours was interesting. Phage given directly into the uterine cavity was later found in foetal blood. Five attempts to demonstrate phage passage across the placental barrier after intravenous administration of non-filtered lysate were unsuccessful.

Non-filtered phage lysates cleared only by centrifugation were used. Since these lysates undoubtedly contained a few living bacteria containing phage which could have passed from mother to foetus in an infectious manner, experiments were performed using lysates which had been freed of all bacteria by filtration (Table I). Exp. 2 and 3 show that phage in sterile filtrates passed from mother to foetus.

Since *Escherichia coli* is a Gram-negative rod, T phage lysates contain Gram-negative endotoxins. It was possible that these toxins could affect foetal membranes so that they became more permeable to phage. Toxic effects were indeed manifest in death of embryos next to site of injection, provided assay time was delayed a few hours or more after inoculation. However, in rats injected in one uterine horn only, normal foetuses at the anterior end of uninjected horn were as likely to contain phage as foetuses in the injected horn. Dead foetuses were not used. To exclude the effects of endotoxin on passage, phage which had been washed and purified by high speed centrifugation was used (Exp. 4, Table I). Intracardiac and intrauterine injections of these preparations showed passage of phage from mother to foetus. In Exp. 5 (Table I), T1 coliphage which is larger than T3,

TABLE I. Recovery of T1 and T3 Coliphages Injected into Pregnant Rats.

Exp. No.	Inoculation	Time (hr)*	Sample tested		Phage recovery
	(T3 non-filtered lysate, titer: 10^{10})				
1	3 ml intracardiac	3½	Maternal blood	(1)†	Positive
			" peritoneal fl.	(1)	"
			Foetal blood	(1)	"
	5 ml into left uterine horn	6	Maternal blood	(1)	"
			Foetal "	(2)	"
	4 ml *Idem*	6½	" "	(4)	"
	4 ml "	23	Whole foetuses	(1)	"
			Maternal spleen	(1)	"
	(T3 filtered lysate, titer: 1.7×10^9)				
2	3 ml into left uterine horn	4	Foetal blood	(5)	3 pos, 2 neg
	3 ml *Idem*	6	*Idem*	(3)	2 " , 1 "
			Foetal fluids	(1)‡	Positive
	2 ml "	6	*Idem*	(1)	"
			Whole foetuses	(1)	"
	(T3 filtered lysate, titer: 1.7×10^9)				
3	5 ml into left uterine horn	24	Foetal blood	(4)	Negative
	2 ml into each uterine horn	24	*Idem*	(4)	3 pos, 1 neg
	(Washed T3 phage, titer: 9×10^9)				
4	1 ml intracardiac	5	Foetal blood	(3)	2 pos, 1 neg
	1.5 ml into each uterine horn	5½	*Idem*	(3)§	1 " , 2 "
	1 ml *Idem*	6	"	(6)	Positive
	2 ml into left uterine horn	7	Whole foetuses	(2)	"
	2 ml into right uterine horn	8	Foetal blood	(3)	1 pos, 2 neg
	(T1 filtered lysate, titer: 10^9)				
5	4 ml into left uterine horn	17	Foetal blood	(4)§	Positive
	4 ml *Idem*	18	Whole foetuses	(1)	"

* Time between inoculation and sampling.
† Numbers in parentheses indicate No. of separate samples tested.
‡ Refers to a mixture of amniotic and exocoelomic fluids.
§ Samples were tested after foetuses had been delivered by the mother.

was inoculated into pregnant rats. This phage also was recovered from foetuses. T phages crossed the placental barrier in 74% of all samples tested after intrauterine injection of the mother.

In Exp. 4 one animal delivered her young 5½ hours after receiving purified T3 phage. The blood of some of these newborn animals contained phage. Also, blood from foetuses born 17 hours after the mother had been injected with T1 filtered lysate, contained phage (Exp. 5, Table I).

In further studies, 6 pregnant rats were inoculated with D29 mycobacteriophage and 5 animals with D32 mycobacteriophage (Table II). After intravenous injection these phages were not found across the placental barrier. Following uterine injection, phage activity was found in 6 out of 14 samples in the case of D29 and in 3 out of 9 samples in the case of D32. No deleterious effects on foetuses were observed when mycobacteriophages were injected into the uterine horn.

Discussion. Apparently, high concentrations of phage must reach the uterine cavity before passage into the foetus becomes evident. Therefore, the probability of similar large particles reaching the foetus under natural conditions is remote; nevertheless, it is of considerable interest to find that it is possible for them to do so. Because quantitative assays were not performed, the actual numbers of phage particles which passed the placental barrier are not known.

The possibility of contamination of foetal samples with phage from maternal circulation is an important source of error in such studies. Even when phage was injected into the uterine lumen it could later be recovered from samples of maternal blood. Therefore, ex-

TABLE II. Recovery of D29 and D32 Mycobacteriophages Injected into Pregnant Rats.

Exp. No.	Inoculation	Time (hr)*	Sample tested		Phage recovery
		(D29 non-filtered lysate, titer: 5×10^9)			
1	3 ml intravenous	4½	Maternal blood	(1)†	Negative
			" peritoneal fl.	(1)	"
			Foetal blood	(3)	"
	4 ml "	7½	Maternal blood	(1)	Positive
			" spleen	(1)	"
			" peritoneal fl.	(1)	Negative
			Foetal blood	(1)	"
	Idem	24	Maternal spleen	(1)	"
			" peritoneal fl.	(1)	"
			Foetal blood	(5)	"
		(D29 filtered lysate, titer: 8×10^8)			
2	3 ml into right uterine horn	5	Amniotic fluid	(1)	Negative
			Foetal blood	(5)	1 pos, 4 neg
	2 ml into each uterine horn	6	*Idem*	(4)	3 " , 1 "
	2 ml into left uterine horn	6½	Amniotic fluid	(2)	1 " , 1 "
			Foetal blood	(1)	Negative
			Whole foetuses	(1)	"
		(D32 filtered lysate, titer: 3×10^8)			
3	2 ml into left uterine horn	4	Whole foetuses	(2)	1 pos, 1 neg
	Idem	4½	Foetal blood	(2)	1 " , 1 "
	"	5	*Idem*	(2)	Negative
	"	5½	Amniotic fluid	(1)	"
			Foetal blood	(2)	1 pos, 1 neg
	"	6	*Idem*	(1)	Negative

* Time between inoculation and sampling.
† Numbers in parentheses indicate No. of separate samples tested.

treme care had to be exercised during sampling to avoid spurious results. The possibility of contamination and use of inoculation routes other than uterine may explain some of the conflicting results of earlier workers.

These experiments do not provide information on the mechanism of phage passage. However, it appears likely that simple diffusion across membranes may be ruled out since electron microscopic examination of the yolk-sac membrane and placenta(11) has not shown pores which would allow passage of such large particles. The electron micrographs have shown abundance of microvilli at the free border of epithelial cells of yolk-sac splanchnopleure. It is probable that passage of phage particles is accomplished by some active method of transport, possibly by pinocytosis of membrane cells.

Since bacteriophage is infectious only when intact, it can be concluded that these particles reached the foetal circulation in an undegraded state. This means that the placental barrier in the rat does not prevent passage of particles as large as D32 mycobacteriophage.

Summary. T1 and T3 coliphages and D29 and D32 mycobacteriophages were injected into pregnant rats. Examination of foetuses and foetal fluids showed presence of phage, which demonstrates that intact particles of relatively large size can cross the placental barrier in the rat. Phage injected into maternal blood rarely reached the embryo. However, when phage was inoculated into the uterine lumen, passage to the foetus was demonstrated in three-fourths of test samples using coliphage, and in one-third of samples using mycobacteriophage. It appears that large concentrations of phage in the vicinity of yolk-sac splanchnopleure are required before passage becomes apparent; this suggests that passage of similar particles from mother to foetus does not frequently occur in nature.

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Metabolic Studies of Vitamin B_{12} (Depinar) (24886)

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When a large dose of Vit. B_{12} is given parenterally, a major portion is excreted in urine and therefore is not available for metabolic functions. Hence, it is desirable to explore any preparation containing cyanocobalamin which reduces such rapid excretion. This report presents evidence that both in experimental animal and man, Vit. B_{12} administered by either intraperitoneal or intramuscular route as its tannate derivative (Depinar) is excreted much more slowly in urine and maintained at significantly higher levels in blood than when B_{12} is given in aqueous form. Data are presented to demonstrate that Vit. B_{12} Co^{60}-tannate administered to the rat results in increased organ content of the radioactive component and presumably due to Vit. B_{12} molecule itself(1).

Methods. Animal experiments. Ten male adult rats of McCollum strain were divided into 2 groups of approximately equal average weight. One group was administered 0.84 μg of Co^{60} B_{12}-tannate (Co^{60} "Depinar"). The second received 0.84 μg of Co^{60} B_{12} (both of 180 μc/mg specific activity) intraperitoneally. Animals were housed in individual metabolism cages. Urine was collected daily under toluene. Urine samples and washings were evaporated to 50 ml on steam bath in graduated 100 ml brown bottle. After 20 days the 10 rats were sacrificed. Liver, kidney and intestines were preserved in frozen state and subsequently were individually homogenized in stainless steel cup on Waring Blender. Radioactivity of urine samples and of total organ homogenates was measured by well scintillation counter. *Human Vit. B_{12} serum levels.* Male, healthy adults were administered either 500 μg of crystalline Vit. B_{12} or 500 μg of Vit. B_{12} as its tannate derivative (Depinar). Blood was obtained by venipuncture prior to administration as well as 8, 24, 72, 144, 216, 504 and 672 hours (4 weeks) after administration of Vit. B_{12} or "Depinar." Serum obtained by centrifugation was stored in frozen state until assayed for Vit. B_{12} by microbiologic assay with *Lactobacillus leichmannii* ATCC 4797 according to method of Skeggs and co-workers(2). All urine was collected for duration of study and aliquots were analyzed at 6, 24, 72, 96, 124, 144 and 168 hours by above technic. *Materials.* "Depinar" is a Vit. B_{12} tannic acid derivative prepared and kindly supplied by Thompson & Hecht of Armour & Co. Radioactive Vit. B_{12}, Co^{60} Vit. B_{12} (Co^{60} B_{12}) preparations employed for these tests were kindly supplied by Dr. Chas. Rosenblum of Merck & Co. Specific activity of 180 μc/mg. "Resting" cells of *Lactobacillus leichmannii* ATCC 4797 was described previously(3).

Results. In Fig. 1, urinary excretion of

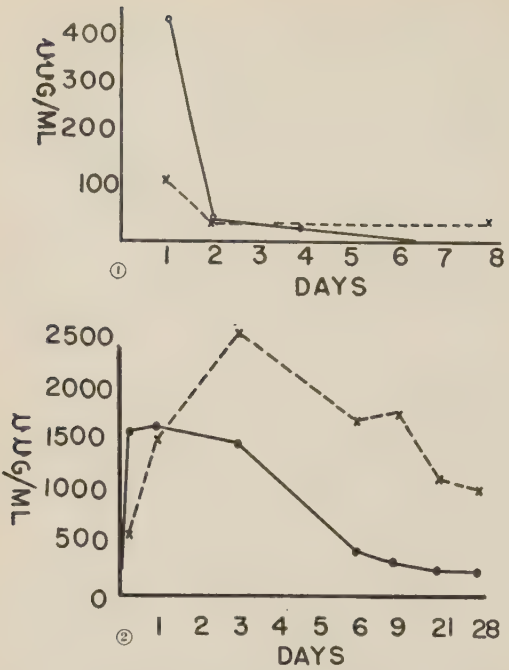

FIG. 1. Urinary excretion of radioactive vit. B_{12} and "Depinar" administered to rats intraper. Each point represents avg value of 5 rats receiving 0.84 μg vit. B_{12} or "Depinar"/100 g body wt. ○—○ Vit. B_{12} Co^{60}. ×—× "Depinar" Co^{60}.

FIG. 2. Serum vit. B_{12} levels of human subjects receiving cyanocobalamin or its tannate derivative "Depinar." ●—● Vit. B_{12}. ×—× "Depinar."

Co^{60} Vit. B_{12} and Co^{60} "Depinar" is plotted as function of time. Noteworthy is the initially large difference between Co^{60} Vit. B_{12} and Co^{60} "Depinar" treated groups. Each value represents average Vit. B_{12} level expressed in mμg/daily urine sample of 5 rats administered 0.84 μg of vitamin/100 g body weight intraperitoneally. The Co^{60} Vit. B_{12} group excreted an average of over 400 mμg during first day's collection period, whereas Co^{60} "Depinar" group slightly over 100 mμg. The second day urinary output of radioactive Vit. B_{12}, as measured as Co^{60} activity, was essentially equal for both groups. However, the Co^{60} "Depinar" treated group leveled off at approximately 35 mμg and remained at that level until at least the 8th day. The Co^{60} Vit. B_{12} treated group excretion declined sharply so that very little radioactivity appeared in urine after 4th day.

In Table I, radioactivity in selected rat organs, namely: liver, kidney and intestine, is tabulated. Comparison is made of these organs removed 20 days after intraperitoneal administration of 0.84 μg of Co^{60} Vit. B_{12} or Co^{60} "Depinar"/100 g body weight. These organs were removed from the same 10 animals employed for the urinary excretion study. Each value represents average of 5 animals.

In Group A (Vit. B_{12} treated animals) average body weight 215 g; radioactivity of the 3 organs analyzed was consistently lower than Co^{60} "Depinar" group (Group B). The greatest differences observed were in kidney and intestines; these levels being 106.2 $\pm$ 19 and 245.6 $\pm$ 4.2 mμg/total kidney and 29.4 $\pm$ 5 and 69.6 $\pm$ 7 mμg/total intestines, respectively for Co^{60} B_{12} and Co^{60} "Depinar" groups. Liver and kidney were chosen as target organs since Harte, *et al.*(4) previously demonstrated persistence of significant storage of Vit. B_{12} at least to several months in these 2 organs and easily detectable amounts after 20 days.

In Fig. 2, Vit. B_{12} serum levels of human subjects receiving cyanocobalamin (Vit. B_{12}) or its tannate derivative ("Depinar"), are plotted against hours after administration. With intramuscular administration of Vit. B_{12}, serum level, as measured by modification (5) of method of Skeggs, *et al.*(2) employing *L. leichmannii* ATCC 4797, reached maximum level of approximately 1700 $\mu\mu$g/ml at end of first day. After 3 days, the level was slightly

TABLE I. Radioactivity in Organs of Rats Administered Co^{60} Vit. B_{12} and "Depinar."

Group	Treatment	Body wt (g)	mμg vitamin activity/total organ		
			Liver	Kidney	Bowels
A	Vit. B_{12}	215	85.8 $\pm$ 28.8*	106.2 $\pm$ 19	29.4 $\pm$ 5
B	"Depinar"	200	100.6 $\pm$ 6.4	245.6 $\pm$ 42	69.6 $\pm$ 7

Organs removed 20 days after intraper. administration of 0.84 μg/100 g body wt of Co^{60} tagged vit. B_{12} or "Depinar."

Each value avg of 5 animals (rats/serum).

* Stand. dev.

TABLE II. Excretion of Vit. B_{12} Activity in Urine of Subjects Receiving Intramuscular Cyanocobalamin or Its Tannate Derivative.

Group	Treatment	Hr after administration*						
		6	24	72	96	120	144	168
A	500 μg cyanocobalamin	300	72	—	—	—	—	—
B	500 μg vit. B_{12}-tannate	—	—	.79	.472	.32	.363	.104

— = No appreciable vit. B_{12} activity. 5 subjects were used in each study.
* Expressed as total γ/B_{12}.

less than 1500 $\mu\mu$g/ml, then it fell rapidly to basal level (380 $\mu\mu$g/ml). At 8 hours, a near maximum serum level was obtained with Vit. B_{12} (1600 $\mu\mu$g/ml) which was in marked contrast to serum level of 550 $\mu\mu$g at 8 hours of subjects given "Depinar." This was approximately equal to basal level. With intramuscular administration of "Depinar" there was no large increase in serum level of Vit. B_{12} activity until the end of first day (1600 $\mu\mu$g/ml). Vit. B_{12} activity in serum increased until end of third day (over 2500 $\mu\mu$g/ml) and started to decline so that by end of sixth day, when the B_{12} group had already returned to basal level, the "Depinar" treated group remained at high serum level (1600 $\mu\mu$g/ml). This level did not change significantly during next 3 days. At the end of 21 days, serum Vit. B_{12} activity was still elevated (approximately 1100 $\mu\mu$g/ml) and even after 28 days approximately 850 $\mu\mu$g/ml at which time experiment was terminated. Vit. B_{12} activity of first day serum samples of both groups was approximately equal (1700 $\mu\mu$g/ml). At this time, mean serum value of soluble Vit. B_{12} group began to decrease, whereas "Depinar" groups continued to increase markedly for the next 48 hours. At end of 3 days and until ninth day, there was a constant and sustained difference of approximately 1100 $\mu\mu$g/ml between the 2 groups.

Table II tabulates appearance of Vit. B_{12} activity in urine of human subjects receiving intramuscular cyanocobalamin or its tannate derivative "Depinar." Subjects receiving 500 μg cyanocobalamin, Group A, excreted significant amounts of Vit. B_{12} in urine the first 72 hours. Vit. B_{12} activity appeared in the 6 hour urine sample, persisted until 72nd hour, then no significant activity could be detected by microbiological technics. In Group B, the first urine specimens of subjects receiving "Depinar" equivalent to 500 μg cyanocobalamin contained no appreciable Vit. B_{12} activity. Such activity appeared in appreciable amounts in the 72-hour sample and persisted until at least 168th hour after administration, when collection of urine samples was discontinued.

Discussion. When Vit. B_{12} is administered at 500 μg or 1000 μg level intramuscularly to man almost all vitamin activity appears quickly in urine(6). In view of the use of other biological complexes to enhance body retention and increase blood levels of the substance in question (over the soluble form), "Depinar" was prepared. This is an insoluble complex of Vit. B_{12} and tannic acid which breaks down at slow rate to free Vit. B_{12} in the body. It was believed that if Vit. B_{12} could thus be provided in depot form so that it would be excreted at a slower rate and thus provide a continuous source of this vitamin for metabolic function, conservation and fuller utilization of Vit. B_{12} could be achieved.

Our data offer evidence that prolonged Vit. B_{12} activity was obtained. Table I, Fig. 1 and 2 indicate that urinary output of Vit. B_{12} activity is markedly decreased initially and sustained for considerably longer period of time when Vit. B_{12} is administered as "Depinar."

In view of prolonged retention of Vit. B_{12} when offered as its tannate derivative, it would be of interest to evaluate this Vit. B_{12}-tannate complex in management of hyperglycemia and glycosuria induced by prolonged injection or hypersecretion of cortisone. In diabetics with retinopathy, it has been reported(7) that such patients excrete significantly more of test intramuscular dose of Vit. B_{12} than nondiabetic subjects and secrete significantly more cortisone than diabetics without retinopathy. These patients likewise have elevated serum Vit. B_{12} levels. Increased urinary excretion and serum levels may well be the re-

sult of hypertrophy of the adrenals with resultant decrease in ability of tissue to retain Vit. B_{12}. Further investigation is necessary to ascertain whether "Depinar" would be retained to clinical advantage of these patients.

Conclusions. 1. When rats were administered "Depinar" and soluble Vit. B_{12} both tagged with Co^{60}, the group receiving the tannate derivative of Vit. B_{12} had elevated radioactivity in liver and markedly elevated radioactivity in both kidney and intestines, assumed to be due to B_{12} Co^{60}-tannate complex. 2. This material was likewise excreted much more slowly in urine of both rats and man than aqueous Vit. B_{12}. 3. Serum Vit. B_{12} activity of normal human subjects administered "Depinar" was significantly greater and sustained a much longer time than those receiving soluble Vit. B_{12}. 4. Whether Vit. B_{12} administered as tannate derivative is of any metabolic benefit over the soluble form to patients requiring supplements of this vitamin is yet to be investigated.

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Build-Up and Regression of Inhibitory Effects of Cholic Acid on *in vivo* Liver Cholesterol Synthesis.* (24887)

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That certain bile acids inhibit *in vivo* both synthesis and degradation of rat liver cholesterol has been demonstrated(1,2). Whenever dietary cholic acid inhibited liver cholesterol synthesis, there was a small but very significant increase in liver cholesterol level. It is well-known that increases in liver cholesterol levels are attended by decreases in liver cholesterol synthesis rates(3,4,5). Therefore, does cholic acid directly inhibit rate of liver cholesterol synthesis or does it initiate a change in level of liver cholesterol which in turn brings about the inhibition? The present studies were undertaken to clarify the mechanism of action of cholic acid by determining the order of changes in bile acid, cholesterol and phospholipid concentrations, in relation to changes in *in vivo* liver cholesterol synthesis rates. These studies have been made during early build-up and regression phases of effects of cholic acid.

Methods. Exp. A. Thirty-two Sprague-Dawley female albino rats weighing 250-280 g, were maintained *ad lib.* on basal diet consisting of 97% Rockland rat ration and 3% corn oil for 2-wk observation. The animals were then divided into 2 groups. Controls continued on basal diet *ad lib.,* while treated group received basal diet supplemented with 0.5% cholic acid. During experimental period, controls and treated animals consumed 17.8 $\pm$ 3.10 and 17.4 $\pm$ 1.86 g/day respectively. After dietary periods of 12 hours, 1, 2, and 3 days, 4 control and 4 treated rats received intraperitoneal injections of 50 μc of acetate-1-C^{14}. Six hours later the rats were sacrificed and samples of blood and liver removed for analysis. *Exp. B.* Thirty rats similar to those used in Exp. A were maintained on same basal diet. The animals were

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TABLE I. Effect of Dietary Cholic Acid on Serum and Liver Total Cholesterol, and on Serum Bile Acid, as a Function of Time.

	Serum total cholesterol		Liver total cholesterol			Serum bile acid		
	Control	Cholic acid*	Control	Cholic acid*		Control	Cholic acid*	
Exp. period	mg %		mg/g†		P	mg %		P
12 hr	99.0 ± 8.6	98.5 ± 9.7	2.47 ± .08	2.62 ± .33	.43	3.80 ± .36	3.90 ± .06	.66
1 day	80.3 ± 9.1	88.2 ±16.6	2.63 ± .08	3.05 ± .13	<.01	3.79 ± .68	3.64 ± .70	.78
2 days	98.3 ± 7.4	83.0 ± 5.3	2.38 ± .11	3.13 ± .48	.025	3.74 ± .55	7.20 ± .34	<.01
3 "	89.9 ±12.0	98.3 ± .6	2.44 ± .12	3.13 ± .44	.021	4.06 ± .45	7.70 ±1.49	.02

* .5% cholic acid in diet. † Wet wt.

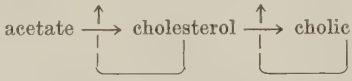
P indicates probability that observed difference between the 2 means is due to chance.

divided into 2 groups. Controls were maintained on basal diet. Treated rats received basal diet supplemented with 0.5% cholic acid for 14 days, after which they were placed on unsupplemented basal diet for the regression study. After regression intervals of 0, 1, 3, 7 and 14 days, 3 control and 3 treated rats were injected intraperitoneally with 50 μc of acetate-1-C^{14} in saline. Six hours following injections, the animals were sacrificed and blood and liver samples removed for assay. In Exp. A and B, serum and liver total cholesterol, serum bile acid, and serum and liver cholesterol-x-C^{14} were determined as previously described (2). In addition, in Exp. A, samples of liver were weighed and dried over P_2O_5. Total lipids were extracted with 1:1 mixture of methanol and chloroform, and determined gravimetrically. Aliquots of extracts were used for total cholesterol(2) and phospholipid(6) determinations.

Results. Exp. A. Relationship of changes in liver composition to decrease liver cholesterol synthesis effected by cholic acid, are presented in Tables I, II, and III. Serum bile acid levels became elevated by second day of experiment (Table I). Previous results(2) show that this level remains constant for an additional 12 days of cholic-acid feeding. Liver total cholesterol (Tables I and II) was significantly elevated in one day and reached a constant level in 2. Liver moisture content and total lipid (Table II) did not change during experiment. On the other hand, total phospholipid decreased significantly during the 3-day build-up period. Incorporation of acetate-1-C^{14} into liver cholesterol (Table III) started to drop during first day of cholic acid treatment. Synthesis rate continued to decrease through 7th day(2), and remained at that level as long as cholic acid feeding was continued. There were no changes in serum total cholesterol concentrations; however, specific activity of cholesterol-x-C^{14} in serum followed the same pattern as in liver.

Exp. B. Tables IV and V give results on regression of dietary cholic acid effects. Serum bile acids (Table IV) remained elevated through third day of regression, while liver cholesterol levels returned to near normal within 3 days. Serum cholesterol levels remained constant throughout experiment. Incorporation of acetate-1-C^{14} into total liver cholesterol increased exponentially during 14-day regression period. Increasing level of serum cholesterol-x-C^{14} paralleled that in liver.

Discussion. In a previous study(2), it was proposed that inhibition of cholesterol synthesis by cholic acid is probably due to a feed-back reaction:



(7)

This mechanism was suggested because observed increases in liver total cholesterol levels caused by dietary cholic acid were accompanied by simultaneous decreases in liver cholesterol synthesis rates. The possibility remained that changes observed in liver total cholesterol concentration were misleading due to decreases in hepatic moisture content. However, since results presented in Table II show that there were *no* significant changes in moisture content, the increases in liver cholesterol are real. This is further confirmed by results obtained from determination of liver total cholesterol on a dry-weight basis.

Another possible explanation for the increase of liver cholesterol and subsequent

TABLE II. Effect of Dietary Cholic Acid on Rat Liver Moisture Content, Total Lipid, Phospholipid, and Total Cholesterol.

Exp. period	Moisture content		Total lipid		Total phospholipid		Total cholesterol		P
	Control	Cholic acid*	Control	Cholic acid*	Control	Cholic acid*	Control	Cholic acid*	
	%		mg/g†						
12 hr	68.7 ± .52	68.3 ± .27	279 ± 14	310 ± 17	63.4 ± 3.9	67.9 ± 7.8	7.59 ± .26	8.41 ± .37	.04
1 day	69.6 ± 1.92	68.4 ± 1.20	315 ± 15	331 ± 20	62.2 ± 3.9	54.5 ± 6.9	7.60 ± .39	8.31 ± .42	.05
2 days	70.0 ± 2.0	69.4 ± .63	326 ± 41	303 ± 25	55.2 ± 7.0	49.0 ± 4.5	7.60 ± .50	9.21 ± .98	.07
3 "	69.3 ± .60	70.1 ± .60	313 ± 19	297 ± 23	60.5 ± 3.1	48.2 ± 7.1	7.92 ± .16	9.30 ± .34	<.01

* .5% cholic acid in diet.
† Dry wt.
P indicates probability that observed difference between the 2 means is due to chance.

TABLE III. Incorporation of Acetate-1-C^{14} into Liver and Serum Total Cholesterol at Different Time Intervals.

Exp. period	Liver total cholesterol		Serum total cholesterol	
	Control	Cholic acid*	Control	Cholic acid*
	counts/min./mg			
12 hr	3,501	2,943	2,916	2,521
1 day	3,195	2,208	2,983	1,876
2 days	2,875	1,595	2,333	1,378
3 "	3,296	1,368	2,717	1,171

* .5% cholic acid in diet.

slowing of turnover rate has been suggested by Friedman and Byers(8,9). They showed that increased plasma cholesterol by infusion of large amounts of cholic acid is preceded by increased plasma phospholipid levels. However, this mechanism can not be applied to our experiments on liver, in which liver phospholipid decreased to some extent while liver cholesterol increased (Table II).

During feeding of cholic acid (Tables I and III), increase of liver cholesterol concentration was paralleled by simultaneous decrease in liver cholesterol-x-C^{14} activity. Bile acid levels did not rise until after first day of treatment; this was to be expected because serum rather than liver bile acids were being measured.

In the regression study (Tables IV and V), levels of liver cholesterol and serum bile acids returned to control levels by the 7th day. Although liver cholesterol levels receded somewhat more rapidly (2nd - 3rd day) than serum bile acid levels (3rd - 7th day), this is probably due to metabolic changes in cholic acid (10,11) which alter its effectiveness in elevating liver cholesterol. The method employed in determining serum bile acids was non-specific, and would measure bile acid conjugates as well as free bile acids.

Along with regressions of serum bile acid and liver cholesterol, there was an exponential increase in liver cholesterol-x-C^{14} activity. Although the most rapid increase in activity corresponded to first 4 to 7 days of study, activities were still slightly low even at the end of 14 days. These findings are similar to those of Taylor *et al.*(5) who found that feeding cholesterol to rats for 7 days decreased the rate of synthesis in liver slices, and

TABLE IV. Regression of Effects of Dietary Cholic Acid on Cholesterol Metabolism in Rat Serum and Liver.

Exp. period (days)	Serum total cholesterol		Liver total cholesterol			Serum bile acid		
	Control	Cholic acid*	Control	Cholic acid*	P	Control	Cholic acid*	P
	mg %		mg/g†			mg %		
0	81.0 ± 4.0	91.1 ± 5.0	2.53 ± .25	3.56 ± .50	.02	3.07 ± .64	6.97 ±1.91	.03
1	92.8 ±11.5	89.1 ± 6.8	2.42 ± .25	2.71 ± .15	.18	3.49 ± .20	4.73 ± .97	.19
3	87.6 ± 7.8	89.0 ±19.7	2.44 ± .20	2.34 ± .13	.51	2.58 ± .17	3.79 ± .55	.02
7	85.9 ± 3.9	88.8 ±13.8	2.34 ± .14	2.35 ± .84	.35	2.79 ± .14	2.74 ± .10	.75
14	95.2 ± 1.5	90.1 ± 4.0	2.43 ± .11	2.43 ± .74	1.00	2.82 ± .39	2.75 ± .65	.90

* .5% cholic acid in diet. † Wet wt.

P indicates probability that observed difference between the 2 means is due to chance.

that recovery to near normal but slightly low rates of synthesis took from 6 to 8 days. Unfortunately these authors made no attempt to correlate decreasing liver cholesterol levels with increasing synthesis rates. If the evidence is considered as a whole, there are indications that the proposed feed-back reaction is operating with cholic acid. Whether other bile acids would act in the same way is open to question and is being investigated.

Summary. A study of sequence of events during initiation and regression of inhibition of cholesterol synthesis by dietary cholic acid was made in rats. In the initiation study, increases of liver cholesterol and serum bile acid levels paralleled decreases of liver cholesterol-x-C^{14} activity. There was a decrease in liver phospholipid during the same time interval. In the regression study, serum bile acid and liver cholesterol returned to control levels more rapidly than the rate of liver cholesterol synthesis. The results suggest that dietary cholic acid initially elevates liver cholesterol, which in turn leads to the inhibition of acetate-1-C^{14} incorporation into liver cholesterol.

TABLE V. Regression of Effects of Dietary Cholic Acid on Incorporation of Acetate-1-C^{14} into Liver and Serum Total Cholesterol.

Exp. period (days)	Liver total cholesterol		Serum total cholesterol	
	Control	Cholic acid*	Control	Cholic acid*
	counts/min./mg			
0	2,361	894	2,366	854
1	3,154	1,135	2,800	1,009
3	2,895	1,659	2,549	1,395
7	3,148	2,126	2,823	1,808
14	3,525	2,715	2,721	2,126

* .5% cholic acid in diet.

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Protective Action of Cysteinamine (β-Mercaptoethylamine) Against X-Irradiation-Induced Sterility in CF_1 Male Mice.* (24888)

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It has been demonstrated(1,2) that cysteinamine (β-mercaptoethylamine), an amine containing an –SH radical, is efficacious in affording protection to mice exposed to otherwise lethal doses of x-rays. Rugh and Wang (2) showed that in a group of male CF_1 mice given cysteinamine intraperitoneally prior to a single 100% lethal dose of 700 r whole-body exposure of x-rays, 67% survived for the 30-day observation period. It is the purpose of this investigation to determine whether cysteinamine has an equally protective effect against x-ray induced sterility in CF_1 male mice.

Materials and method. The albino mice used were of the CF_1 strain, 2 months of age, and weighed approximately 25 g each at time of x-irradiation. They were randomly divided into groups and treated as shown in Table I. The groups in Table I consisted of: (a) normal male controls; (b) males receiving whole-body exposures of 625 r x-irradiation; (c) males receiving exposures of 700 r x-irradiation limited to the testes; and (d) males receiving pre-treatment of cysteinamine and whole-body exposures of 700 r x-irradiation. Cysteinamine was given *via* the intraperitoneal route in a dose of 3 mg (in 1 ml physiological saline solution), 5 to 15 min. prior to irradiation. Those mice which received whole-body exposure to x-rays were irradiated 5 at a time in a perforated, cylindrical plastic cage measuring 13.25 cm inside diameter and 4 cm inside height. Calibration of the x-irradiation was made in air with the ionization chamber in the center of a closed cage. Constant movement of the mice within the cage tended to minimize the effect of any small difference in concentration of x-irradiation that might exist between the center and the periphery of the cage, all of which was well within the exposure field. The mice receiving 700 r to the testes only, were anesthetized with 0.25 ml of 0.3% pentobarbital sodium given intraperitoneally, These mice were then pinned to a board, covered with a lead shield that protected all of the body except the testes and posterior extremities, and exposed to x-rays 5 at a time. In all cases the distance from the target to the center of the mouse's body was 30 cm. Other physical constants of x-irradiation were: Westinghouse Quadrocondex constant-potential x-ray machine run at 210 KV, 15 MA, filtration 0.28 cu and 0.50 Al, and a dose rate of 265 r per min. in air. Exposure time for mice receiving a total exposure of 625 r was 2′ 22″, and for those receiving 700 r, 2′ 39″. Following x-irradiation, the animals were placed 5 in a cage, then returned to the animal rooms where they received the usual care, with food and water *ad libitum*. A daily record of deaths recorded for a period of 30 days. Then, the surviving mice were mated as follows: one x-irradiated male with 2 normal females per cage, and similar pairing for the normal controls. After mating, the mice were checked daily for fertility for a period of 9 months. The criterion for fertility was the production of litters by one of female mice in the cage for each of the 9 successive 30-day periods. Each litter was counted, weighed, recorded, then removed from the mother. The experiment was terminated when the mice reached 12 months of age, in order that natural reproductive senility should not influence the fertility records.

Results. Fertility records of the males are summarized in Table I. At no time were any of the experimental groups completely sterile, although there were differences in per cent fertility among the 4 groups. During the first 30-day mating period, fertility rate was 71.4% among mice pretreated with cystein-

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TABLE I. Mortality and Fertility Rates in Male Mice X-irradiated under a Variety of Conditions.

Period	Normal controls		625 r whole-body irrad.		700 r to testes		Cysteinamine + 700 r whole-body irrad.	
Mortality	No. surv. / No. orig.	% surv.	No. surv. / No. orig.	% surv.	No. surv. / No. orig.	% surv.	No. surv. / No. orig.	% surv.
30 days	38/40	95	60/279	21.5	80/90	88.9	46/70	65.7
Fertility (30 day periods)	No. fert. / No. surv.	% fertile	No. fert. / No. surv.	% fertile	No. fert. / No. surv.	% fertile	No. fert. / No. surv.	% fertile
1	34/38	89.5	27/59	45.8	30/78	38.5	15/21*	71.4
2	36/37	97.3	50/57	87.7	26/77	33.8	18/20	90.0
3	34/37	91.9	43/54	79.6	60/77	77.9	16/20	80.0
4	"	"	34/51	66.7	66/74	89.2	13/18	72.2
5	31/37	83.8	29/41	70.7	66/74	89.2	11/18	68.8
6	27/37	73.0	25/35	71.4	57/73	78.1	8/13	61.5
7	19/20*	95.0	18/28	64.3	43/47*	91.5	8/ 9	88.9
8	16/19	84.2	17/25	68.0	39/46	84.8	7/ 9	77.8
9	13/19	68.4	12/22	54.5	37/45	82.4	7/ 9	77.8

* Some mice in these groups were discarded by mistake, and were not tested for fertility.
Surv. = survival; orig. = original; fert. = fertile.

amine which survived 700 r whole-body exposure. This high fertility rate is not significantly different from that for the normal controls (P greater than 10% using χ^2 test corrected for continuity). On the other hand, during the same mating period, there is a significant difference between normal controls and mice which received only 625 r x-irradiation without cysteinamine pretreatment as well as those which received 700 r to the testes only (P less than 1%). There is also a significant difference between the cysteinamine pretreated group receiving 700 r whole-body irradiation and the untreated group with irradiation only to the testes (P less than 5%).

By the end of 90 days post-irradiation, the untreated group which received 625 r whole-body irradiation had fertility rates not essentially different from those observed in the group pretreated with cysteinamine and irradiated with 700 r over the whole body. On the other hand, the untreated group which received 700 r to the testes only showed recovery in fertility during the third period, or 120 days post-irradiation. During these periods, there is no significant difference in fertility rates among all groups.

Discussion. When administered prior to x-irradiation, cysteinamine has been shown to be very effective in increasing percentage of survival of mice given otherwise lethal doses of x-rays(1,2). Our data (Table I) on percentage of survival is well in accord with that reported by the above authors; 65.7% of the males pretreated with cysteinamine survived 30 days after whole-body x-ray exposure of 700 r. Of the males pretreated with cysteinamine which survived exposure to 700 r, 71.4% proved to be fertile 60 days after irradiation; the difference in per cent of fertile animals between this group and the normal controls is not significant. At the same time, the per cent of fertile males was significantly below normal (P less than 1% for both groups) in those groups which were not pretreated with cysteinamine and received, respectively, dose of 625 r total-body exposure and 700 r directly to the testes. The former of these 2 groups returned to normal 90 days after being x-rayed, while the latter did not return to normal until 120 days after x-irradiation (Fig. 1). Thus, the most important finding in this investigation is that cysteinamine is capable of preventing the transient sterility in the first 2-3 months following x-irradiation given to the whole body or to the testes alone.

Rugh and Wolff(3) found that whole-body x-irradiation of 50 r will sterilize all CF_1 female mice within a period of 30 weeks, and that 100 r will sterilize all the mice within a period of 8 weeks. They(4) also found that cysteinamine injected prior to x-irradiation with 50 r, prolonged average fertility expectancy by 11.42 weeks, but did not prevent

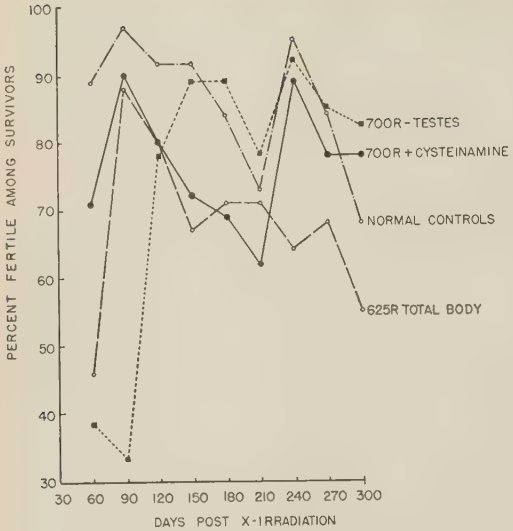

FIG. 1. Fertility records for males.

eventual sterilization even at this low dosage. Their preliminary experiments using 100 r indicated that this level of exposure was so high, and onset of sterilization so rapid, that it would be difficult to demonstrate the protective effect of any drug.

In male mice, there ensues following x-irradiation a brief period of fertility attributable to the presence at time of exposure of resistant mature sperm(5). Since our males were not mated until 30 days post-irradiation, we could not observe whether cysteinamine had any effect on this period of fertility. Following x-irradiation, there occurs a maturation depletion period(6), *i.e.*, progressive loss of spermatogenic cells, beginning with spermatogonia and progressing in order of developmental sequence until reduction in number of spermatozoa occurs(7). This "maturation depletion" leads to a period of sterility owing to the fact that the maturation process has been interrupted.

Following this period of sterility, if the animal does recover fertility, the time of its return depends on dose and species used. Eschenbrenner and Miller(8) showed that in mice given a whole-body exposure of 400 r, one finds spermatozonia, spermatocytes and a very few spermatozoa 4 weeks after x-irradiation; and at 6 weeks after exposure the tubules of the testes contain all spermatogenic elements from spermatogonia to mature spermatozoa, although the population is not yet normal in numbers. Snell(9) demonstrated that in 2 males x-rayed with 800 r, motile sperm had reappeared in the epididymides by 20 weeks after treatment, and then when one of these males mated with 2 normal females, each female gave birth to a litter 16 weeks after treatment. The similar mating of a male x-rayed with 600 r resulted in litters at 26 and 28 weeks after treatment. It can be calculated from our data that for CF_1 males, it takes up to 70 days for total recovery to occur after a whole-body exposure of 625 r, and 100 days after 700 r irradiation given to the testes. The time span accords with that reported in the literature.

Investigators agree that the spermatogonia are the most radiosensitive of the spermatogenic elements, and that owing either to the effect of x-rays in inhibiting mitosis in the spermatogonia(8,10,11) or to the fact that irradiation causes their death(7), there is no restoration of the spermatogenic elements until the spermatogonia are replenished. It would appear from our observations on the mice x-irradiated with 700 r and pretreated with cysteinamine, that cysteinamine is capable of protecting the pre-spermatogenic elements from x-ray damage. It is thought possible that the effective –SH carrying compounds may combine temporarily with some radio-sensitive enzymes, thus making them radio-resistant(4).

Summary and conclusions. Cysteinamine, when administered prior to x-irradiation, has been shown to be very effective in increasing percentage of survival of CF_1 mice given otherwise lethal doses of x-rays. It is also capable of preventing transient sterility in male mice during first 2 or 3 months following x-irradiation given to whole body or to testes alone.

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A Method for Investigating Net Water Fluxes Across Individual Proximal Tubules.* (24889)

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In attempting to evaluate possible mechanisms involved in transport of materials by the kidney, it would be useful to know the kinetics of the transport processes. Experiments estimating rates of water movement across the tubules of Necturus have been carried out by Schatzmann *et al.*(1), using a perfusion system. It is the purpose of this report to describe a simple method for determining net water fluxes across tubules of rat kidneys.

Methods. Micropipettes were drawn with a Brinkman Micro-puller. They were half filled with a test solution by gentle heating of the broad end and plunging the still hot pipette into a beaker containing the test solution. As the air within the pipette cooled and contracted, the solution was drawn into the pipette. The pipette was then mounted in the pipette holder of an Aloe microinjection apparatus. Oil was then forced in the open end of the pipette, expelling the remaining air through the micro-orifice, thereby filling the remainder of the pipette. The microinfusion apparatus is shown in Fig. 1. A one cubic

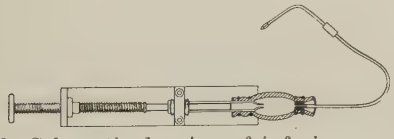

FIG. 1. Schematic drawing of infusion apparatus.

centimeter syringe was mounted on a brass rack which held a threaded rod for adjusting delivery rate. A short length of rubber pressure tubing was wired to the syringe and the other end of the pressure tubing was wired to an adapter which was in turn connected to the pipette holder by means of plastic tubing. The whole system was filled with mineral oil. Upon pushing in the syringe plunger, the pressure tubing inflated and thereby acted as a pressure reservoir providing a relatively constant rate of infusion. Some pressure drop undoubtedly takes place as delivery proceeds but since the volume delivered is very small compared to total volume of the system (less than one part in 2000), pressure falls caused by fluid infusion can be neglected. To avoid leakage around the syringe plunger, tight fitting tuberculin syringes were selected, and no effect of leakage on infusion rate has been detected. A control experiment whereby delivery rate of solution was determined as a function of time is shown in Fig. 2. In this experiment, time required for the oil-solution meniscus to move between the major divisions of an ocular micrometer was determined as the infusion apparatus delivered solution into a pool of fluid held in a planchet. The volume delivered was estimated from measurements of the pipette dimensions. Rate of delivery was purposely made higher than that to be used in biological experiments since it was thought that at high delivery rates the chance of detecting inadequacies in the system would be enhanced. To determine infusion rate, movement of the oil-solution interface was monitored with a telescope mounted on a microscope. The fine adjustment knob on

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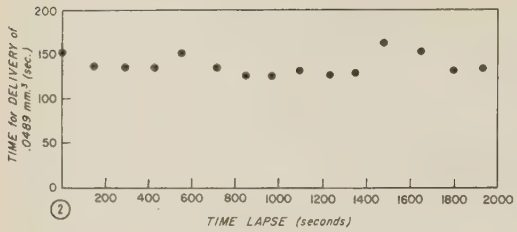

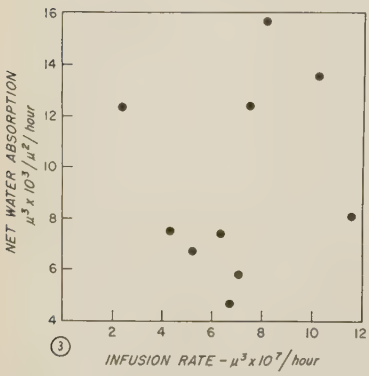

FIG. 2. Plot of delivery rate during continuous delivery showing relative constancy of flow.

FIG. 3. Plot of absorptive rate as function of infusion rate showing lack of correlation between these 2 factors.

one side of the microscope was removed and a 360° protractor mounted in its place. The fine adjustment of the microscope was calibrated by determining number of degrees turn necessary to move the telescope cross hair one mm. In the apparatus used in these experiments, one mm was equal to 2160 degrees of turn. Repeat measurements were reproducible to within 30°. Rats were prepared for the experiments according to methods previously described(2) with the modification that mineral oil was layered over the peritoneal Ringer bath to improve visualization of the kidney. Tubules were first impaled with an oil filled pipette in order to fill the whole nephron with heavy mineral oil. Before filling, however, a first test was done to localize the proximal loop. A small droplet of oil was injected into the lumen, and watched as it was swept through the tubules. Time required for the droplet to pass through a proximal surface loop is less (less than a second usually) than that required to pass through a distal loop (more than 1.5 seconds usually). A second evaluation of localization of the proximal portion was made during the filling procedure. Most surface nephrons do not have any distal representation. These were most often found and identified by observing that filling was rapid and continuous and that the oil filled loops formed a relatively compact mass of tubules. When the tubule had a surface distal representation, filling of the loops occurred in 2 phases, a rapid filling of the tubules around the puncture site followed at an appreciable time later by a filling of a second group of tubules, usually somewhat displaced from the first group. In the experiments to be described, the original puncture was always made in a proximal loop. A mineral oil filled loop other than that by which the filling was accomplished was selected for study and impaled with a micropipette containing the test solution. Test solution was then infused at a slow rate (.01 to .1 cmm per hour). A bubble of test solution formed in the tubular lumen which grew somewhat in size over the first few minutes, then stopped growing. When the "steady state" size was reached, net rate of water removal across the walls of the tubule in contact with the solution bubble was equal to rate of infusion. Dimensions of the intraluminal droplet were measured with a Spencer ocular micrometer, and knowing the infusion rate, it was possible to calculate the net flux per square micron of luminar area.

Results. In a series of 10 determinations, using Ringer solution as the test solution, net rate of water absorption ranged from 5-15 $\times$ 10^3 μ^3/μ^2/hr. The mean was 9.3 with a standard error of 1.3 $\times$ 10^3 μ^3/μ^2/hr.

The possibility should be considered that the water which leaves the tubule does so because of filtration rather than by tubular activity. One would then expect that infusion rate would be related to pressure, and that if the water showed bulk movement through the tubular wall because of high hydrostatic pressure, the derived net flux rate would be proportional to rate of infusion. Fig. 4 shows a plot of absorption rate as a function of infusion rate, and it can be seen that net flux values are scattered about an average value instead of being positively correlated with infusion rate.

It may be argued that in formation of the

solution droplet, the oil is not moved from the tubular walls, and that the area over which absorption takes place is less than that which is measured because of the presence of oil microdroplets. One of the more striking features of the solution droplet is the fact that it is sharply curved inward. This shows that the tubule walls are strongly hydrophilic. It does not seem reasonable therefore, that interference by residual oil is likely. Tubule hydrophilia may, however, be responsible for introducing an error into the results. Because the contact angle is small, the effective area over which water movement is taking place may actually be greater than that which is measured, thereby causing overestimation of the reabsorption rate.

Two kinds of control experiments have been done. In animals which have died during preparation for an experiment, a steady state size has not been observed to be attained within a surface loop at a time which is at least 10 minutes after time of death. This shows that water movement had been depressed. When infusion solution was lightly colored with trypan blue, it was seen that color intensity of the intratubular droplet increased as time passed. These experiments showed that removal of fluid did not result from infusion solution slipping past the oil column. It has now become standard practice to color slightly the infusion solution with dye to make the solution droplet more apparent. Furthermore, if excessive damage is done in the puncturing process, intense local staining of the cells around the pipette tip is usually observed. As might be expected, certain proximal cells show some cellular staining(3).

Summary. A method has been described for measuring net rate of water movement across single tubules of rat kidney *in situ*. Water is reabsorbed at a rate of 9.3 ± 1.3 μ^3/μ^2 proximal tubular surface/hr. Possible criticisms of the method have been discussed.

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Secretion of Cholesterol by Intestinal Mucosa in Patients with Complete Common Bile Duct Obstruction.* (24890)

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A mixture of endogenous and exogenous cholesterol is absorbed from the lumen of intestine. The endogenous portion (total secretion) normally consists of one fraction secreted by liver in bile and a second secreted by the intestine. It has been determined in normal man under standard conditions of food intake that there is a total secretion of approximately 2 g/day of cholesterol into the gut(1,2, unpublished). The method by which these results were obtained is summarized below. Two other groups of investigators, one using a special balance method(3), the other by determining dilution of fed C^{14}-cholesterol in chyle collected from urinary fistula(4), have obtained similar results. Such determinations indicate the *sum* of quantities secreted in bile and by intestinal mucosa since in normal man contributions from the 2 sources can not be separated. This paper reports results of such studies in 3 patients with complete obstruction of common bile duct due to carcinoma of the head of the pancreas. In these patients the fraction usually gaining access to gut *via* the bile had been excluded so that all of endogenous cholesterol in the lu-

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TABLE I. Results of Intestinal Cholesterol Exchange Studies in 3 Patients with Complete Biliary Obstruction Due to Carcinoma of Head of Pancreas.

			Cholesterol					
				Excretion		Absorption		
Patient	Age	Sex	Intake, mg/day	C^{14}, %	Total, mg/day	C^{14}, %	Total, mg/day	Secretion, mg/day
J.J.	49	♀	45	91.5	400	8.5	37	392
J.P.	47	♀	60	63	292	37	171	403
O.M.	73	♀*	45	>99	300	<1		255

* This patient had diarrhea during the study. The others had formed stools throughout.

men had been secreted by intestinal mucosa. Under these conditions intestinal secretion equals total secretion and quantification of the latter is equivalent to measurement of intestinal secretion. Determination of quantity of cholesterol secreted by intestinal mucosa, either in animals or in man, has not previously been reported.

Methods and material. The method was previously described(1,2). In brief, each patient was fed a low-cholesterol, semi-synthetic liquid diet containing an inert nonabsorbable indicator; to food fed the second day a small quantity of free C^{14}-cholesterol was added. Fecal excretions of unabsorbed C^{14}-cholesterol and of stable cholesterol were estimated with the aid of inert indicator and verified by calculations of the sum of the quantities of these compounds contained in total collections of stools. It was assumed that free C^{14}-cholesterol fed in food and the cholesterol secreted into gut lumen were metabolized in the same manner and that the same proportions of each that had originally been present in intestinal contents were excreted in the stools. Under these conditions size of total cholesterol "pool," or total cholesterol available for absorption (TCAA) $= \frac{C_s}{C_1} \times 100$, where C_s is rate of stable cholesterol excretion (g/day) in stools and C_1 is percentage of administered C^{14}-cholesterol unabsorbed and excreted in the stools. TCAA-intake = daily secretion into the gut. Our studies were carried out immediately preoperatively in 3 patients with carcinoma of the head of pancreas that led to obstruction of the common bile and pancreatic ducts. In all 3, absence preoperatively of urobilinogen in urine and observations at operation indicated that obstructions to the biliary and pancreatic ducts were complete. Examination of surgical specimens removed from 2 patients confirmed this; resection was not done in the third patient (O.M.).

Results. In these 3 patients (Table I) fecal excretion of unabsorbed ingested C^{14}-cholesterol was large and varied from more than 99% in O.M. who had diarrhea, to 63% in J.P. Concurrently the stable cholesterol excretion varied from 292 to 400 mg/day. As diminution of absorption of stable cholesterol was assumed to correspond to that of C^{14}-cholesterol it was calculated that from 255-403 mg/day had been secreted into the gut.

Discussion. As expected if the major source of cholesterol secreted into the gut were bile, under these conditions there was marked diminution of secretion from normal values of 2 g or more to 400 mg or less/day. As evidence was quite definite of completeness of obstruction to bile flow into the intestine, the conclusion that these reduced quantities of cholesterol were "secreted" solely by mucosa was justified. These amounts were evidently the sum of secreted cholesterol plus quantity of this sterol contained in desquamated epithelial cells. It is not known how abnormalities that accompanied obstruction of the common bile duct (complete exclusion of bile and pancreatic secretion from intestine; general impairment of digestion and absorption; increased serum concentrations of bile acids, bilirubin and free cholesterol; decreased serum concentration of ester cholesterol) influenced normal mucosal secretion of cholesterol. However, it has been established that a constituent of pancreatic secretion, presumably cholesterol esterase, and normal enterohepatic circulation of bile are both necessary for normal transfer of cholesterol from lumen of gut to the chyle(5). As cholesterol absorptive

function was demonstrably markedly deranged, presumably as a result of abnormalities induced by exclusion of bile and pancreatic secretion from the gut, the secretory function of the mucosa may also have been modified. Hence no conclusions as to quantity of cholesterol normally secreted by the mucosa can be drawn.

The sum of biliary and intestinal cholesterol secretions totals more than 2 g/day in normal persons under our conditions(1,2). If mucosal secretion normally were not greater than 400 mg/day, found in 2 of our subjects then there is more than 1.5 g/day of cholesterol normally secreted in the bile. This is a larger quantity than is usually estimated from studies carried out in bile fistula patients (6). Results obtained by the latter method may vary from normal, because of error in estimating the proportion of bile diverted into the fistula, because of partial exclusion of bile from the gut, and because studies were carried out in patients with diseases of the biliary tract.

Summary. In 3 patients with complete obstruction of biliary and pancreatic ducts cholesterol "secretion" by intestinal mucosa was 250-400 mg/day. Quantification of this function in either man or animals has not been reported previously.

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The Dwarf Mouse—An Animal with Secondary Myxedema.* (24891)

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It has been concluded that dwarfism in the house mouse is inherited as a recessive Mendelian characteristic dependent upon a single gene(1). Dwarfism results from hypofunction of the hypoplastic pituitary gland(2,3). It has been reported that there is an almost complete lack of eosinophil cells in the anterior pituitary, accompanied by deficiency in growth hormone production(2,3). Snell(4) first demonstrated that thyroid of the dwarf mouse was defective. Later(2,3) morphology of dwarf-mouse thyroid was investigated more thoroughly. Smith and MacDowell(2) and Kemp and Marx(3) found that thyroid tissue was interspersed with adipose and fibrous connective tissue which made it impossible to dissect and weigh the gland as a separate organ. Gland tissue present showed signs of delayed function. In addition, 30-40% lower basal metabolism occurred in dwarf mice as compared to normal mice of same age(5). A histological study of the thyroid from a few thyrotropin-stimulated dwarf mice showed no structural changes to indicate increased activity(3), but by daily transplants of fresh rat pituitaries into dwarf animals, small body size, sterility, and associated endocrine abnormalities, including thyroid changes of hereditary dwarfism, were corrected(2). All available data suggest secondary deficiency of the thyroid, but which pituitary hormone insufficiency is responsible has not been clarified. With modern technics, it is possible to obtain more exact information as to the cause of thy-

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roid disorder involved in the complex picture of dwarfism in mice. This report gives data that the dwarf mouse, besides other deficiencies, is in a state of secondary myxedema, dependent at least partly upon lack of thyrotropic hormone. This is of value in further investigations of pituitary cell morphology and in experiments concerned with effect of thyrotropin on connective tissue.

Material and methods. The 40 dwarf and 30 normal mice were from same litters and kept together in same room at 22°C. Dwarf animals were fed a special diet prepared from pellets given to normal mice. The pellets were ground and mixed with milk to make them easier to eat. Ten dwarf mice were killed by decapitation and blood from the wound was pooled for protein-bound iodine (PBI) determination.‡ A similar procedure was repeated with 10 other dwarf mice for a second PBI determination. Blood from 10 normal siblings obtained in same way, was analyzed for PBI content for control purposes. Ten intact dwarf mice and 10 dwarf mice treated with thyrotropin (Organon, 0.16 U.S.P./0.25 mg in 0.25 cc of water/animal) were injected subcutaneously with 10 μc of radioactive iodine I^{131}§ in 0.1 cc of carrier solution (0.01 μg I^{127}/μc I^{131}). Twenty-four hours later radioactivity was measured over neck and pelvic regions with EKCO‖ scintillation counter N 559 A used with auto-scaler N 530 and provided with special animal holder. The distance from animal to crystal was 50 mm. There was an aperture 21 mm in the chimney-like lead shield 30 mm thick. The same procedure was carried out with 10 control and 10 thyrotropin (Organon, 0.66 U.S.P./1 mg in 0.25 cc of water/animal) stimulated normal mice. Activity in each region was measured 3 times in each animal and averages used in calculating results.

Results. PBI values for dwarf mice (1.2 and 1.3 μg/100 ml of serum) compared with those obtained in blood from normal siblings (7.3 μg/100 ml of serum) showed a low pathological level.

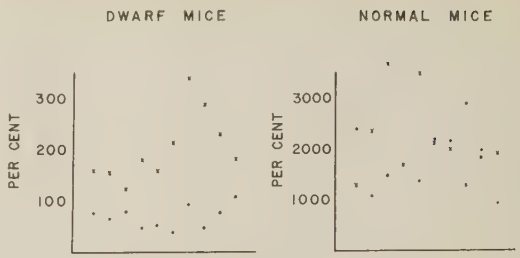

FIG. 1. Radiation from neck in % of radiation from pelvic region. ● Control animals. × Thyrotropin treated animals. (Each point represents individual animal.)

Results from radioactive iodine uptake test are shown in Fig. 1. Thyroids of untreated dwarf mice did not accumulate iodine. In 9 out of 10 animals, activity over pelvic region was higher than that over the neck. It is easily seen that thyrotropin stimulated the thyroid to increased uptake of iodine in all treated dwarf animals. Untreated mice took up about 10 times as much radioactive iodine as untreated dwarf mice. Normal animals also increased their uptake after thyrotropin stimulation, but response was not as uniform as in dwarf animals.

Conclusion. The histological picture(2,3), low basal metabolism(5), inability of thyroid to accumulate iodine, and low PBI values shown in present study, indicate a myxedematous state in dwarf mice. Daily rat pituitary transplantations into dwarf animals restore thyroid to normal(2), from which is concluded that a pituitary factor, or factors, is responsible for the poor state of the dwarf thyroid. Thyrotropin is able to stimulate the thyroid gland of dwarf mice, and sensitivity seems greater than in normal siblings. It may be concluded that there is a lack of both thyrotropin and of somatotropin in the dwarf mouse. These mice are in a state of secondary myxedema and an important etiological factor for this disorder is thyrotropic pituitary hormone deficiency.

Summary. 1) The level of protein-bound iodine in serum of dwarf mice, determined in 2 groups of 10 each (1.2 and 1.3 g/100 ml), was very low in comparison to the value (7.3 g/100 ml) obtained on serum of 10 normal

‡ Kindly carried out by Dr. Th. Friis at Frederiksberg Hospital, Copenhagen, Denmark.

§ Joint Establishment of Nuclear Energy Research, Norway.

‖ EKCO Works, Southend-on-Sea, Essex, England.

siblings. 2) Thyroids of untreated dwarf mice did not show accumulation of radioactive iodine (I^{131}), whereas injection of thyrotropin (0.16 U.S.P.) significantly stimulated the glands to iodine uptake. 3) From these data, and previous observations, it is concluded that the dwarf mouse is a secondary myxedematous animal because of deficient production or complete lack of thyrotropic pituitary hormone.

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Distemper and Measles Viruses. I. Lack of Immunogenic Crossing in Dogs and Chickens.* (24892)

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The demonstration of distemper antibodies in human serum and gamma globulin suggested at first an infection of humans with distemper virus at an early age, with possible clinical involvement of a respiratory nature (1,2). Although the presence of these antibodies in humans was confirmed(3) and their time of appearance in children studied(4), the question as to whether distemper virus *per se* was responsible for human illness has remained unanswered. More recently, a possible relationship between measles and distemper viruses was presented as another likely explanation for the appearance of distemper antibodies in humans(5,6,7). An investigation was therefore initiated in this laboratory to study the suggested similarity between the 2 viruses by a variety of laboratory methods. This communication reports on the search for measles and distemper antibodies in sera from puppies and chickens immunized with distemper virus, and in those of chickens which received multiple injections of measles virus.

Materials and methods. Virus strains. The canine distemper (CD) strain used for both immunization of puppies and chickens and for neutralization tests was the Lederle chick-embryo-adapted CD virus(8). Virus stock consisted of a 30% infected chorio-allantoic membrane (CAM) suspension which was maintained in frozen state. The 50% chick-embryo-infective titer (CED_{50}) was $10^{3.2}$-$10^{3.7}$ per ml. Puppies were challenged with a suspension of Snyder Hill strain CDV(9) prepared and stored as described earlier(10). A pool of Edmonston strain† measles virus (MV), having 50% tissue-culture, infective titer (TCD_{50}) of $10^{6.3}$/ml, was used for immunization of chickens. It represented a second monkey kidney passage of chick-embryo-tissue-culture adapted virus(11). Serum neutralization was carried out with a pool of the same MV strain derived from human-amnion tissue culture. This pool had a TCD_{50} titer of $10^{3.5}$-$10^{4.5}$/0.1 ml and was prepared in HeLa cells following 5 consecutive passages of the virus in this laboratory in an established human amnion cell line and one passage in HeLa. *Tissue culture.* HeLa cultures used for the propagation and titration of MV, and in the MV neutralization test, were grown in Eagle's solution(12) containing 20% calf serum. Just before inoculation, these cultures were renewed with the same solution containing 5% calf serum. *Control immune sera. CD.* A susceptible puppy was injected subcu-

* The authors wish to express their appreciation to Marie Murrin for technical assistance and to E. Manoogian for help in preparation of manuscript.

† We are indebted to Dr. John F. Enders of Children's Hospital, Boston, for both lines of Edmonston strain of measles virus.

taneously with 2 ml of the 30% infected CAM suspension described above. It was bled out 5 weeks later and its serum was stored in the freezer. Repeated titrations of the serum gave neutralizing titers from 1:1000 to 1:7000 against 20 to 200 CED_{50} of CDV. *MV.* This serum was obtained from a cynomolgus monkey which, in the course of another experiment, had been simultaneously injected 1) intracerebrally with 1 ml of human-amnion-tissue-culture propagated Edmonston strain MV and 2) intramuscularly with 200 mg cortisone acetate. Repeated titrations of this serum gave neutralization indices of 316 to 3,160. *Neutralization tests. CD.* Five-fold dilutions of serum and approximately 100 CED_{50} virus suspension were mixed and incubated for 4 hours at 4°C. Each mixture was then inoculated in 0.2 ml amounts on the CAM of six 7-day-old chick embryos by Gorham's technic (13). After 7 days incubation at 35-36°C, the large top of each egg was removed and the CAM examined for lesions indicative of CD infection. Serum neutralization titers (SN_{50}) were calculated by Reed and Muench's formula(14). Titration of virus stock was carried out with each set of tests. *MV.* Undiluted normal and test sera were each mixed with equal volumes of undiluted MV, and incubated for 30 min. at room temperature. Residual virus in each mixture was titrated as follows: 10-fold dilutions were made in pH 7.2 phosphate buffer and 0.1 ml of each dilution inoculated into each of 2 HeLa culture tubes. The tubes were incubated at 37°C and examined for 14 days for the appearance of multinucleated giant cells characteristic of measles. Renewal medium was changed every 4 days. Neutralization indices were derived from the difference between virus titers in presence of normal control and test sera. All sera were routinely inactivated at 56°C for 30 minutes before use in neutralization tests. Normal and immune serum were included with each set of CD antibody titrations, and an immune serum control with all measles tests. *Complement-fixation (CF) test with MV.* CF antigen consisted of a HeLa culture virus pool which was concentrated 3 to 4 times by dialysis in the cold against undiluted, buffered glycerine. It was used in the test at a dilution of 1:4. The test was carried out by mixing 0.1 ml of 2-fold serum dilutions with 0.1 ml antigen (2 units) and 0.2 ml complement (2 units). After incubation in a 37°C water bath for one hour, 0.2 ml sheep cells sensitized with 2 units of hemolysin were added and a reading was made after further incubation for 20 minutes at 37°C. Under these conditions, the immune monkey control serum gave titers between 1:128 and 1:256.

Results. Immunization of puppies with CD virus. Of a litter of nine 10-week-old mongrel puppies, 6 were each injected subcutaneously with 2.0 ml of 30% CD-infected CAM suspension. Five weeks later, 3 of the vaccinated and the 3 unvaccinated animals were each inoculated intravenously with 1.5 ml of a 10% dog brain suspension containing about 1500 50%-lethal doses (LD_{50}) of Snyder Hill strain CD virus. All immunized puppies, including those which were challenged, remained normal throughout the observation period. In contrast, non-vaccinated puppies which were given virulent CD virus developed signs of the disease; one recovered, one had a prolonged illness and was sacrificed when moribund 44 days after inoculation and the third died of distemper on the 25th day. All puppies were bled at the time of vaccination and 4 to 5 weeks later. Bleedings were also obtained from challenged animals as follows: 8-10 weeks post challenge from those previously vaccinated as well as from the surviving control; on the 21st day from the control that died and on the 35th day from the sacrificed animal. Results of neutralization tests for CD and measles antibodies with all collected serum specimens are summarized in Table I. It is seen that all puppies, save the one that died of distemper, developed appreciable levels of CD antibodies. Titers were of the same order following vaccination alone or after vaccination and challenge. In contrast, no measles antibodies were detected in any animals either after vaccination or following challenge.

Hyperimmunization of chickens with CD or MV. Two groups of six 7-months-old Peterson strain Cornish Red Cross roosters, weighing approximately 8 lbs each, were inoculated, one with CD and the other with MV according to the following schedule: Day 0—

TABLE I. Results of Testing of 9 CD Immune Puppies for CD and Measles Neutralizing Antibodies and for Measles CF Antibodies.

Immunization status	Challenge outcome	CD-serum neutralizing dose 50 (SND_{50}) Pre-vaccin.	Pre-chall.*	8-10 wk post-chall.	Measles antibodies Pre-vaccin. CF	Pre-vaccin. NI	Pre-chall. CF	Pre-chall. NI	8-10 wk post-chall. CF	8-10 wk post-chall. NI
Vaccinated	Not challenged	<1:5	1:1400		<1:4	0	<1:4	0		
"	"	"	1:1200		"	0	"	0		
"	"	"	1:1400		"	0	"	0		
"	Remained normal	"	"	1:1400	"	0	"	0	<1:4	0
"	"	"	1:940	1:280	"	0	"	0	"	0
"	"	"	1:1600	1:8600	"	0	"	0	"	0
Not vaccinated	Sickened—sacrificed 44th day	"	<1:5	1:330 †	"	0	"	0	"	0
"	Sickened—recovered	"	"	1:400	"	0	"	0	"	0
"	Sickened—died 25th day	"	"	1:10 ‡	"	0	"	0	"	0
CD immune dog serum control		1:1,000 to 1:7,000			CF = <1:4		NI = 0			
Measles immune monkey serum control		Not tested			CF = 1:128-1:256		NI = 315 to 3,150			

* These bleedings were obtained 4 to 5 wk following vaccine admin. † Serum drawn on 35th day after challenge. ‡ Serum drawn 4 days before death.

After bleeding, each chicken was injected intramuscularly with 2.5 ml of virus-adjuvant mixture (5 parts virus suspension; 1 part Arlacel, (mannide monooleate), 4 parts Bayol F (mineral oil)) into each of 4 sites, and 1 ml of adjuvant-free suspension intravenously. Day 7—Each chicken received 1 ml virus suspension intravenously. Day 21—Day 0 schedule was repeated. Day 31—All chickens were bled. CD virus consisted of either 10 or 20% CAM suspension, whereas MV was administered as undiluted tissue culture fluid. Results of neutralization tests for CD and measles antibodies on sera taken on Days 0 and 31 are given in Table II. It is obvious that response was strictly specific to the antigen injected; all 6 chickens of the first group developed CD antibodies, and only one of the second group failed to respond with measles antibodies.

Discussion. The suggestion by others that CD and MV may possibly share related antigenic constituents stimulated great interest on the part of the present authors. A strain of CD virus had been adapted to chick embryos in this laboratory(8) resulting in a modified agent, totally avirulent but greatly immunogenic for its natural hosts. Should measles and CD virus be truly immunologically related, attempts to immunize man against measles with modified CD virus could have a chance of success.

Under the conditions of the experiments reported above, no relationship could be demonstrated between the two viral agents. Chick-

TABLE II. Serum Neutralizing Antibodies of 12 CD or MV Hyperimmunized Chickens.

Hyperimmunized with	Measles: NI* Pre-vaccin.	Measles: NI* 31 days after 1st inj.	CD: SND_{50} titers† Pre-vaccin.	CD: SND_{50} titers† 31 days after 1st inj.
Canine distemper	0	0	<1:5	1:940
	0	0	"	1:125
	0	0	"	1:420
	0	0	"	1:240
	0	0	"	1:55
	0	0	"	"
Measles	0	0	<1:5	<1:5
	0	315	"	"
	0	"	"	"
	0	"	"	"
	0	>315	"	"
	0	"	"	"
CD immune dog serum control	0		1:1,000 to 1:7,000	
Measles immune monkey serum control	315 to 3,150		Not tested	

* NI = Neutralization index.

† SND_{50} = Serum dilution causing neutralization of virus in 50% of inoculated chick embryos.

ens were selected for a parallel study of both viruses because they are not known to be susceptible to natural infection by either. In contrast, dogs injected experimentally with MV would be constantly under the threat of a natural CD infection, sometimes even under the most careful isolation conditions. Extension of this study to monkeys and ferrets is in progress, as is the testing of sera from humans who received injections of either an experimental measles vaccine or the chick-embryo-adapted strain of avirulent CD virus.

Summary. Puppies, vaccinated against canine distemper (CD) or immunized and challenged with CDV, developed high levels of homologous antibodies but failed to develop CF or serum neutralizing antibodies for measles virus (MV). Similarly, chickens hyperimmunized with CD virus did not develop measles neutralizing antibodies, although CD antibodies in appreciable levels appeared in 6 of 6 birds. Hyperimmunization of another group of chickens with MV was followed by no neutralizing CD antibodies in any of 6, whereas 5 of 6 birds gave a substantial measles response.

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Effects of Amines and Monoamine Oxidase Inhibitors on Hypothalamic Succinic Dehydrogenase Activity.* (24893)

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Although the mammalian hypothalamus appears to modulate certain activities of the anterior lobe of the hypophysis, the mechanisms remain obscure. Contributions by amines to stimulation of parts of the hypothalamus and perhaps hypothalamo-hypophyseal transmitter systems appear likely, due to: (A) the especially high concentration of certain of these amines as well as monoamine oxidase in the hypothalamus(1,2); (B) pharmacologic evidence of a close connection between central sympathetic stimulation and turnover of diencephalic and mesencephalic sympathin (3); and (C) elevation of posterior hypothalamic electrical activity, respiration and aerobic glycolysis following epinephrine administration(4,5) and elevation of supraoptic and paraventricular acetylcholinesterase and acid phosphatase after administration of serotonin(6). The work reported here employs succinic dehydrogenase system as an anaerobic test system for evaluating effects of physiologically significant amines, related compounds and monoamine oxidase inhibitors. Succinic dehydrogenase activity increases during functional stimulation in several glandular and protein-synthesizing tissues. The present work aims to determine whether this enzyme activity in the hypothalamus responds to amines and drugs possibly active in

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regulating or modifying hypothalamic function *in vivo*.

Methods and materials. Adult rats (Long-Evans strain), golden hamsters (*Mesocricetus auratus,* Waterhouse), and deer mice (*Peromyscus maniculatus,* Wagner) born and raised in our laboratory were used. The deer mice are from 2 inbred strains derived from feral individuals taken in Washtenaw County, Mich. (= W-M) and San Benito County, Calif. (= SB-C). Within each experiment, littermates were evenly distributed, and in rats the age range was less than 2 weeks. During each experiment animals were individually caged and maintained at 22-26°C with 14 hours of light/day; food and water were available *ad lib.* All injections were intraperitoneal and all experiments contained control groups injected with the solvent, water or 0.85% sodium chloride. Untouched controls were included as well in some experiments. Succinic dehydrogenase activity was measured by reduction of 2, 3, 5-triphenyl-2H-tetrazolium chloride (Eastman) by the enzyme system and colorimetric determination of the resulting formazan. This method is derived from that of Seligman and Rutenberg(7) and subsequent authors. The anterior and posterior halves of the hypothalami were immediately dissected from rapidly decapitated animals and were cut into small pieces while in a drop of 0.85% sodium chloride. Pieces from each area were rinsed for exactly 15 minutes at 24-26°C in 4 ml pH 8.25 phosphate buffer + sodium cyanide (1:2 dilution of solution 1 below) to remove endogenous substrates, and then incubated at 37°C with mild agitation for 2 hours in 1.5 ml of freshly prepared medium containing equal parts of the following 3 stock solutions and adjusted to pH 8.25: (1) 0.225 M sodium phosphate buffer, 0.1% sodium cyanide; (2) 5.4% sodium succinate, .01 M $AlCl_3$, .001 M $CaCl_2$; and (3) 0.4% tetrazolium. Incubations in control media (lacking succinate) failed to produce significant amounts of formazan in the tissue and minor variations in size of tissue fragments were without significant effect. After incubation, tissues were rinsed in distilled water, fixed in 10% neutral buffered formalin, dried overnight at 37°C on previously weighed aluminum foil pans, weighed to nearest μg with quartz helix balance (Microchemical Specialties Co., Berkeley), and extracted with ethyl acetate and grinding. The extracted formazan was rapidly measured at 490 mμ and the μ moles of tetrazolium reduced/μg of dry tissue were computed by method similar to that used by Shelton and Rice(8). The 2 sides of the anterior and posterior hypothalami of rats and hamsters were determined separately and later averaged before the average of each experimental group was computed.

Results. Monoamine oxidase inhibitors: 1-isonicotinyl 2-isopropyl phosphate (Marsilid or Iproniazid) at 170 mg/kg body weight (20 ♀ rats, 15-16 hr) and at 30 mg/kg (20 ♂ rats, 20-21 hr) produced no change in hypothalamic succinic dehydrogenase activity (HSD). Likewise in deer mice (W-M ♀) this drug was ineffective both alone (200 mg/kg, 18-21 hr) and when followed by 5-hydroxytryptophan (15 mg/kg, 5-7 hr), which may pass the blood-brain barrier and raise the brain level of serotonin under these conditions(2). The more potent and longer-acting inhibitor, beta-phenylisopropylhydrazine hydrochloride (JB-516 or PIH)(9) significantly depressed HSD in both rats and deer mice (Table I). In hamsters doses of 30-50 mg/kg produced severe behavioral agitation, but significant change in HSD did not occur, even when 5-hydroxytryptophan was injected 5-6 hr prior to autopsy. The sympathetomimetic amine, amphetamine, which differs from PIH in having an amino in place of the hydrazino group and which has been considered an inhibitor of amine oxidation (10), failed to modify HSD in ♂ rats (N = 47, 25-30 mg/kg) either 18-20 or 5-6 hours later. Reduction of HSD by PIH (20-30 mg/kg) in rats was not significantly modified when followed 5-8 hours before autopsy by 5-hydroxytryptophan (18 mg/kg), 1-3, 4-dihydroxyphenylalanine (7 mg/kg), 1-epinephrine bitartrate (2.5 mg/kg), or 1-norepinephrine (1.5-2.0 mg/kg). Similarly, in deer mice (SB-C) dl-5-hydroxytryptophan · H_2O, 1-3-4-dihydroxyphenylalanine, or both together (each 16 mg/kg) failed to modify the response to PIH.

TABLE I. Reduction of Hypothalamic Succinic Dehydrogenase after Administration of PIH.

Species	Sex	No. of animals	Time, hr†	Dose, mg/kg body wt	AH	P	PH	P
Rats	♀	23	18–20	0	93.69 ± 2.69*		93.79 ± 2.29*	
		16	"	30	85.82 ± 1.75	<.05	82.33 ± 2.57	<.01
"	"	10	19–20	0	86.76 ± 1.44		82.95 ± 3.93	
		6	"	90	57.60 ± 4.97	<.001	45.17 ± 3.29	<.001
		12	5– 6	90	55.47 ± 1.27	"	51.77 ± 1.66	"
Deer mice (W-M)	♂	10	16–18	0	86.91 ± 3.88		79.91 ± 3.43	
		10	"	65	69.96 ± 3.35	<.01	64.40 ± 2.04	<.01

* Mean ± stand. error of mean. † Time = Hr between inj. and autopsy.

P = Probability based on "Student"-Fisher t test of significance of differences between means. AH = Anterior hypothalamus. PH = Posterior hypothalamus.

Amines: None of the amines tested produced significant changes in HSD. These experiments employed, in rats: 1-epinephrine bitartrate (0.2 mg/kg, 18-20 hr; 2.5 mg/kg, 5-6 hr), 1-3, 4-dihydroxyphenylalanine (6-8 mg/kg, 4-6 hr), 5-hydroxytryptophan (18 mg/kg, 5-6 hr), tyramine monohydrochloride (40 mg/kg, 20 or 6-7 hr); in deer mice (SB-C): 1-epinephrine bitartrate (0.6 mg/kg, 18-20 or 6-7 hr, controls untouched).

Discussion. It appears unlikely that reduction of HSD following injection of PIH is due to increased levels of hypothalamic norepinephrine or serotonin, since our experiments with other monoamine oxidase inhibitors were negative even when precursors of these amines were administered. Nor can this effect of PIH be related readily to general central nervous system excitation since amphetamine did not produce decreased HSD and since in hamsters PIH produced severe agitation without significantly influencing HSD. Our surveys indicate that PIH reduces succinic dehydrogenase activity in some other areas of rat's brain as well. It should be emphasized that doses (mg/kg) of PIH which significantly decreased HSD in the rat are higher in our studies than the reported doses effective in monoamine oxidase inhibition and central nervous system stimulation in investigated mammalian species.

Amines that are known either to reduce "brain" succinic acid oxidation (O_2 uptake measured) *in vitro* (tyramine) or to stimulate posterior hypothalamic respiration and aerobic glycolysis *in vivo* (epinephrine) were not effective in modifying HSD *in vivo* in our experiments. This suggests that certain of the aerobic oxidative enzyme systems may be more sensitive than the succinic dehydrogenase system to the effects of these amines.

Summary. Succinic dehydrogenase activity of anterior and posterior hypothalamic areas (HSD) was measured by the reduction of a tetrazolium salt to its colorimetrically measurable formazan. The potent monoamine oxidase inhibitor, beta-phenylisopropylhydrazine, significantly reduced HSD activity in both anterior and posterior areas of adult rats and deer mice but not of hamsters. Other monoamine oxidase inhibitors, epinephrine and several other amines were without significant effects on HSD.

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Influence of Mixing Time on Determination of Red Cell Volume in Normal and Shocked Rats. (24894)

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There is widespread use of radioactive chromium^{51} tagged red blood cells for measurement of red cell volume(1). Application of this technic to the rat has been described (2,3). Reliability of this method in the rat, as evidenced by blood volume determinations with varied mixing times of the injected, tagged cells, has not been established. Further, reliability of the method has not been established in rats in advanced hypovolemic shock. The purpose of this paper is to report red cell volumes, as determined with Cr^{51} rat tagged red cells, with varied mixing times in normal rats and in rats subjected to hypovolemic shock.

Methods. Details of the chromium^{51} erythrocyte tagging and dilution technics employed have been reported(3). Rat red cells were incubated in isotonic $Na_2Cr^{51}O_4$ solution at 4°C overnight. They were then separated, washed 4 times in isotonic saline and resuspended in their previously separated plasma. One milliliter aliquots of the tagged blood were pipetted into lustroloid tubes and counted in the well of a scintillation counter. Approximately .1 ml heparin and 0.4 ml of the counted blood were injected into the femoral vein. The syringe was rinsed once with water and the rinse put into the counted tube from which the injection aliquot was taken and the tube recounted. The difference in counts was taken to represent counts injected. Upon checking the syringe after the single rinse to determine residual counts which might have been lost, it was found that this represented less than 0.5% of the dose and no correction was made for such an insignificant loss. At varying times after Cr^{51} injection, the left common carotid artery was cannulated with polyethylene tubing and the animals were bled through cannulae. This blood was used for microhematocrit determinations and for isotope counting. Arterial hematocrit was used in all calculations and was determined in duplicate in capillary tubing spun in a Phillips-Drucker microcentrifuge. Albino rats of the Holtzman Farms weighing between 190 and 290 g were used. Weight was taken as the morning weight after food had been removed from the cages at about 5:00 p. m. of the previous evening. In a normal series of animals, mixing times of 5, 10, 20 and 30 minutes after isotope injection were allowed and red cell volumes calculated. Eight animals were used in each time period (Exp. 1). In another series of animals bilateral hind limb tourniquets were applied for 4 hours(4). Three hours after release of the tourniquets, when the animals were in profound shock, the tagged cells were injected. Mixing times of 15, 30 and 60 minutes (Exp. 2) and 5 and 10 minutes (Exp. 3) were allowed. Red cell volumes were then calculated. Again, 8 animals were used in each time period.

Results. Exp. 1. When 5, 10, 20 and 30 minute mixing times were allowed in normal animals, the values for red cell volumes were essentially the same for each mixing time period (Table I). The standard deviations were remarkably stable.

Exp. 2. In the tourniquet shocked animals values for red cell volumes were essentially the same for each mixing time period (Table II). When red cell volumes obtained at 30 minutes in the shocked animals were compared to values obtained in the normal animals in Exp. 1 after the same mixing time, no apparent difference was seen. Statistical analysis also showed low probability ($0.10 < p < 0.20$ by Student "t").

Exp. 3. The fact that mean red cell volumes in shocked and normal animals were not different in Exps. 1 and 2 led to the conditions of the third experiment. Here, determinations of red cell volume were made, using short mixing times (5 and 10 minutes) in shocked groups. An untraumatized control group with a mixing time of 5 minutes was included for direct comparison (Table III). It is apparent that the red cell volume obtained in

TABLE I. Red Cell Volume as Measured in Normal Rats after Various Mixing Times.

Wt (g)	No.	Mixing time (min.)	Hematocrit	Red cell vol (ml/100 g)	P
200.0	8	5	47.8 ± 1.6	2.66 ± .17	
208.5	8	10	48.0 ± 2.0	2.58 ± .10	$.20 < p < .30$
216.8	8	20	47.0 ± 1.6	2.62 ± .17	$.50 < p < .70$
199.6	8	30	46.8 ± 1.2	2.69 ± .15	approaches 1

TABLE II. Red Cell Volume as Measured in Hypovolemic Shock Employing Long Mixing Times.

Wt (g)	No.	Mixing time (min.)	Hematocrit	Red cell vol (ml/100 g)	P
206.2	8	15	66.8 ± 2.3	2.60 ± .13	
193.3	7	30	64.4 ± 1.9	2.51 ± .15	$.20 < p < .30$
199.9	7	60	66.7 ± 1.2	2.58 ± .13	$p = .80$

the normal untraumatized group is within the narrow range established in Exp. 1. It is further demonstrated that in the 2 shocked groups determinations of volume show values indistinguishable from each other and from the normal untraumatized animals.

Discussion. While a 20-minute mixing time allows for reproducible red cell volume determinations in the dog(5), such data are not available in the rat. It is apparent from the data presented that red cell volume determinations measured by radiochromium dilution are reliable and reproducible in the rat. In fact, working in groups of 8 animals the mean values reported are reproducible within a little more than 1% variation. From the data reported, an adequate mixing time for the normal rat is 5 minutes from time of tag injection, which is the shortest mixing time employed in the experiments.

It was surprising to find that in rats in hypovolemic shock, characterized as it is by hematocrit elevation, concomitant increase in blood viscosity and lowered arterial pressure, a relatively short mixing time (15 minutes) for radiochromium tagged cells allowed a reliable red cell volume determination. Since there was no *a priori* knowledge of the order of magnitude of red cell loss into the injured extremities following tourniquet trauma, it did not seem reasonable at first to compare red cell volume of these animals with that found in normal animals. It was felt that by using relatively long mixing times under these circumstances, adequate distribution and dilution of the tagged cells would take place and would allow utilization of this technic in shocked animals. As a result, in this experiment, relatively long mixing times (15, 30, and 60 minutes) were allowed and the red cell volumes compared with each other to determine at what time stabilization had occurred.

The finding that absolute red cell volumes in shocked and normal animals were in the same range then suggested simultaneous comparison of the 2 groups with use of shorter mixing times. These again showed values of essentially the same magnitude. It is true that there is some suggestion from the scatter of the 5 and 10 minutes means in the tourniquet treated rats, as well as a probability of 0.02 in the 5 minute mixing time in the tourniquet treated group, that it might be more reliable to use a 15 minute mixing time in deeply shocked rats.

Summary. Determinations of red cell volume using radiochromium 51 tagged red cells

TABLE III. Red Cell Volume as Measured in Hypovolemic Shock Employing Short Mixing Times.

Wt (g)	No.	Mixing time (min.)	Hematocrit	Red cell vol (ml/100 g)	P
245.8	10	5 (controls)	47.4 ± 1.8	2.66 ± .11	
254.5	9	5 (shock)	65.2 ± 3.0	2.51 ± .15	$p = .02$
263.1	9	10 "	66.7 ± 4.8	2.76 ± .17	$.10 < p < .20$

have been made in normal and tourniquet shocked rats using varied mixing times. Red cell volume determinations were significantly reproducible in the normal rat using mixing times of from 5 to 30 minutes. A 15 minute mixing time in the tourniquet shocked rats allowed significantly reproducible determinations of the red cell volume.

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Nephron Reabsorptive Site for Calcium and Magnesium in the Dog.* (24895)

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The technic of stopped flow, introduced by Malvin, Wilde and Sullivan(1), consists in collecting consecutive small volumes of urine discharged from a mannitol-loaded kidney immediately following release of ureteral occlusion of a few minutes' duration. A plot of concentration of any substance in consecutive urine fractions against ordinal number of the fraction, generally shows that the concentration curve passes through a minimum or a maximum according to whether the substance is reabsorbed or secreted by the nephron. Relative locations of maxima and minima of a group of substances in such a plot constitutes a sequence of transport processes which should correspond to the relative order in which these exist anatomically in the nephron. The validity of any sequence established in this way rests upon its agreement with the sequence established by analysis of fluid obtained directly from various segments of the nephron by micropuncture. Moving toward the glomerulus, direct analysis indicates, in the rat at least, that sodium is reabsorbed strongly in first portions of distal tubule(2) while glucose is reabsorbed and dyes secreted in proximal convoluted tubule(3,4) In the stopped flow analysis as described above, the same sequence is observed: concentration minimum for Na (and K), followed by a minimum for glucose and a maximum for p-aminohippurate secretion, the last 2 occurring simultaneously(1). The above substances, whose anatomical site of transport is at least approximately known, can tentatively serve as reference points in stopped flow analyses for other substances which are transported by renal epithelium and which for one reason or another have not been examined by micropuncture. Application of this concept to locate approximate site of transport of calcium and magnesium in the nephron is reported here. The technic was that described by Malvin *et al.* using dogs as experimental animals. The urine reinfusion technic was employed to attain a steady state of renal function and body fluid composition following appropriate priming(5). Methods for measurement of sodium, potassium, p-aminohippurate, inulin, creatinine, calcium and magnesium are those regularly employed in our laboratory(6-9).

Results. Three successful experiments were in complete agreement. Fig. 1 illustrates results of one experiment. Both Ca and Mg are reabsorbed in the same region of the nephron. The area of reabsorption of these cations appears to coincide with the area of K reabsorption and to coincide either with or lie slightly "proximal" to the area of maximal Na reabsorption. On the other hand, the Ca

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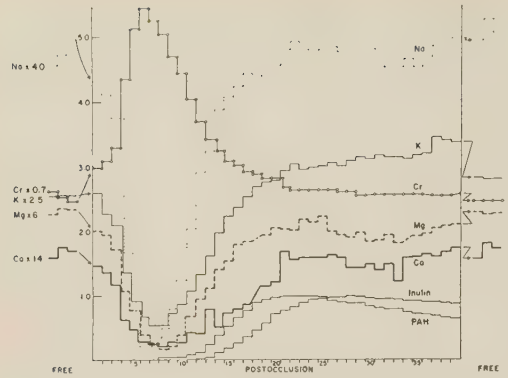

FIG. 1. Electrolyte excretion patterns from a stopped-flow experiment. Creatinine is represented as its U/P ratio, electrolytes (Ca, Mg, Na, K) as clearance ratios to creatinine, and PAH and inulin as fractions of their maximal urinary concentration. U/P and clearance ratios are fitted to arbitrary scale by multiplying by indicated factors. Electrolytes exhibit minima in urine which appear early following release of ureteral occlusion (left) and before appearance of PAH (right), thus relating transport areas to distal segment of nephron. Protocol: Nembutal and chloralose anesthesia; Prime: mannitol 2 g, creatinine 75 mg, KCl 1.5 mM, $CaCl_2$ 0.63 mM, $MgCl_2$ 0.35 mM, dog Ringer-Locke solution 44 ml, all/kilo; equilibration by urine reinfusion 150 min.; 0.6 ml urine samples collected serially following release of 7 min. occlusion; inulin and PAH inj. slowly 2 min. before release.

and Mg minima are "distal" to the region of PAH secretion indicating that these ions are reabsorbed in the "distal" segment of the nephron. The data fail to suggest existence of additional processes of either reabsorption or secretion of Ca or Mg.

A single, distal locus for magnesium transport offers an explanation for the observation of Robinson *et al.*(10) that radioactive Mg appeared only in the "distal" area of a stopped flow graph. Experience with other transport systems, such as sodium and urea in the frog(11,12), indicates that molecules may move opposite to the direction of principal movement while net transport is occurring.

Summary. 1. Stopped flow analysis indicates that calcium and magnesium are reabsorbed strongly in a "distal" region of the nephron approximately coincident with reabsorptive areas for sodium and potassium. 2. The data fail to suggest existence of additional reabsorptive or secretory loci for these ions.

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Homologous and Heterologous Complement Fixing Antibody in Persons Infected with ECHO, Coxsackie and Poliomyelitis Viruses. (24896)

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Although a number of newly recognized viruses recovered from the alimentary tract of man were originally assembled in the ECHO group primarily because they apparently did not belong in already known families of viruses(1), it has become increasingly evident that most of them do share many bio-

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logic properties with each other and with the Coxsackie and poliomyelitis groups of viruses (2). This report will present data on antigenic relationships between these groups of viruses obtained by studying the complement-fixing (CF) antibody responses of persons naturally or experimentally infected with certain ECHO, Coxsackie, and poliomyelitis viruses.

Materials and methods. Sera. Five groups of paired human sera were studied. One group consisted of sera from 11 adults experimentally infected with ECHO type 10 virus (3); a second consisted of sera from 7 persons with naturally acquired ECHO type 16 infections; (these sera were kindly furnished by Dr. F. Neva); a third consisted of sera from 3 adults experimentally infected with ECHO type 20 virus(3); a fourth consisted of sera from 5 adults experimentally infected with Coxsackie B3 virus(3); and the fifth consisted of sera from 6 persons with naturally acquired type 1 paralytic poliomyelitis infections (these sera were kindly furnished by Dr. E. H. Lennette). *Virus strains.* The following virus strains were used to prepare CF antigens: prototype strains of ECHO types 1 through 20,(1,2,4), "Connecticut-5" strain of Coxsackie B1,(5), "Texas pleurodynia" strain of Coxsackie B3, (6) "Powers" strain of Coxsackie B4,(7), "Faulkner" strain of Coxsackie B5,(8), "Mahoney" strain(9) of poliomyelitis type 1, "MEF-1" strain(9) of poliomyelitis type 2, and "Saukett" strain(9) of poliomyelitis type 3. *CF antigens.* ECHO and poliomyelitis antigens, with one exception, were prepared as reported previously(10), except that 2% calf serum was used in place of 1%. In brief, the method was as follows: Rhesus kidney monolayer cultures in 32 oz. prescription bottles with 40 ml of medium consisting of 2% calf serum, 0.5% lactalbumin hydrolysate, and 97.5% Earle's solution were inoculated with virus material so diluted that complete degeneration of tissue did not take place in less than 2 days. After complete degeneration had taken place, the bottles were frozen and thawed once, and the fluids then harvested, and were not centrifuged. Fluids were then stored at approximately –20°C until used as antigen. Multiple freezing and thawing of the antigens was avoided, although no significant decrease in antigen CF titers could be demonstrated after 3 to 4 such cycles. Antigens for ECHO types 4, 5, 6, and 14, and all 3 poliomyelitis types were treated with fluorocarbon before use. Antigens for Coxsackie B viruses and ECHO type 20 were prepared in HeLa cell cultures grown in 32 oz prescription bottles with 40 ml of medium consisting of Eagle's basal medium, plus 10% human serum. After about 6 days, when cultures were very heavy, they were washed 3 times with 40 ml of Hanks' solution, and 30 ml of a medium containing 5% chicken serum, 25% tryptose phosphate broth, and 70% medium 199 was added. Cultures were then inoculated and handled as described above for rhesus kidney cultures. None of HeLa antigens were treated with fluorocarbon. *CF test.* Complement fixation tests were performed by standard technic used in this laboratory(10,11). No antigens were heated. Each antigen in each test was titrated against a standard homologous antiserum, monkey serum for ECHO and poliomyelitis antigens, and mouse serum for Coxsackie antigens. All antigens used had titers of 1:2 or 1:4 and at least 2 units of antigen were used in all tests.

Results. ECHO type 10. Homologous and heterologous CF titers of paired sera from 11 adults who were experimentally infected with ECHO type 10 virus and who subsequently showed a rise in homologous CF antibody are shown in Table I. With one exception, no individuals had a rise in CF antibody against any of the heterologous ECHO, Coxsackie, or poliomyelitis antigens used. Volunteer No. 6, who did show a rise in ECHO type 20 CF antibodies, worked in a hospital where he was in contact with volunteers who had been experimentally infected with ECHO type 20 virus. It was demonstrated that this individual also developed a rise in neutralizing antibody against ECHO type 20.

ECHO type 16. CF titers against various antigens of paired sera from 7 persons with naturally acquired ECHO type 16 infections ("Boston exanthem disease") are shown in Table II. Each pair of sera showed a homologous neutralizing antibody rise and ECHO

TABLE I. ECHO, Coxsackie, and Poliomyelitis CF Antibody Titers of Sera Collected from 11 Persons before and 28 Days after Experimental Infection with ECHO Type 10 Virus.

Date serum collected	ECHO antigens														Coxsackie B antigens		Poliomyelitis antigens		
	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12	E14	E20	B1	B3	P1	P2	P3
7/22/57	<8	<8	16	<8	<8	32	<8	<8	<8	<8	32	<8	<8	<8	—*	—	<8	<8	<8
8/19	"	"	<8	"	"	16	"	"	"	128	16	"	"	"	—	—	"	"	"
7/22	"	"	"	"	"	32	"	"	"	<8	64	"	"	32	—	—	"	"	"
8/19	"	"	"	"	"	"	"	"	"	64	"	"	"	"	—	—	"	"	"
7/22	"	"	32	"	"	16	"	"	"	<8	32	"	"	<8	—	—	"	"	"
8/19	"	"	"	"	"	"	"	"	"	64	"	"	"	"	—	—	"	"	"
7/22	"	"	<8	"	"	<8	"	"	"	<8	"	"	"	8	—	—	"	"	"
8/19	"	"	"	"	"	"	"	"	"	64	16	"	"	<8	—	—	"	"	"
7/22	"	"	"	"	"	"	"	"	"	<8	"	"	"	8	—	—	"	"	"
8/19	"	"	"	"	"	"	"	"	"	64	<8	"	"	<8	—	—	"	"	"
7/22	"	"	16	"	"	"	"	"	"	<8	32	"	"	8	—	—	"	"	"
8/19	"	"	"	"	"	"	"	"	"	64	16	"	"	32	—	—	"	"	"
7/22	—	—	<8	—	"	—	—	—	—	<8	<8	—	—	—	—	—	—	—	—
8/19	—	—	"	—	"	<8	—	—	—	32	"	—	—	—	—	—	—	<8	<8
7/22	—	—	"	—	"	—	—	—	—	<8	"	—	—	—	—	—	—	—	—
8/19	—	—	"	—	"	<8	—	—	—	16	"	—	—	<8	<8	<8	—	<8	<8
7/22	—	—	8	—	"	—	—	—	—	<4	—	—	—	—	—	—	—	—	—
8/19	—	—	"	—	"	<8	—	—	—	16	<8	—	—	<8	<8	<8	—	<8	<8
7/22	—	—	"	—	"	—	—	—	—	<4	"	—	—	—	—	—	—	—	—
8/19	—	—	"	—	"	<8	—	—	—	8	"	—	—	<8	<8	<8	—	<8	<8
7/22	—	—	16	—	"	—	—	—	—	<4	8	—	—	8	—	—	—	—	—
8/19	—	—	8	—	"	<8	—	—	—	8	"	—	—	"	<8	—	<8	<8	<8

* — = not tested.

type 16 virus was recovered from 6 patients. Although only 2 individuals (WO & FD) had a CF antibody rise against the prototype strain of ECHO type 16 virus, 3 other persons (KW, RM, and AR) had CF antibody rises against one or more of the heterologous antigens used. Three individuals (WO, FD, and KW) had CF rises against a number of heterologous ECHO and Coxsackie antigens (ECHO types 2, 5, 6, 7, 8, 11, 14, 20 and Coxsackie B3).

ECHO type 20. CF titers for paired sera from 3 adults who were experimentally infected with ECHO type 20 virus and who showed a rise in homologous CF antibody are shown in Table III. Each individual developed heterologous CF antibodies (including ECHO types 5, 6, and 11, and Coxsackie B types 1, 3, 4, and 5).

Coxsackie B3. CF titers of paired sera from 5 adults who were experimentally infected with Coxsackie B3 virus and who showed a rise in homologous CF antigens are shown in Table IV. Three individuals had rises in heterologous CF antibodies (which included ECHO types 4, 6, 7 and Coxsackie B1).

Poliomyelitis type 1. CF titers against various antigens of paired sera from 6 persons with naturally acquired type 1 paralytic poliomyelitis are shown in Table V. Although it was possible to demonstrate a rise in homologous titer for 5 individuals, none developed a rise in heterologous CF antibodies against the antigens used. Moreover, a rise in poliomyelitis CF antibodies was not noted in persons infected with any of the other viruses in this study.

Discussion. Multiple heterologous CF antibody responses observed in persons infected with certain ECHO and Coxsackie viruses and absence of a predictable group response indicate that this type of serologic test with the type of antigens employed would be of very limited usefulness for type-specific diagnosis of infection by these viruses. It is quite possible, of course, that persons (*e.g.*, young children) undergoing their first infection with a

TABLE II. ECHO, Coxsackie, and Poliomyelitis CF Antibody Titers of Sera Collected from Persons in Acute and Convalescent Phase of "Boston Exanthem" Disease (ECHO 16).

Patient	Date serum collected	ECHO antigens E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12	E14	E16	E20	Coxsackie B antigens B1	B3	Poliomyelitis antigens P2	P3
WO	6/22/54	—*	64	—	—	—	64	<8	16	—	—	32	—	8	<8	64	—	16	—	—
	7/15	<8	128	<8	<8	<8	128	16	64	<8	<8	128	<8	32	64	128	—	64	—	—
FD	6/28	—	8	—	—	<8	8	<8	<8	—	—	8	—	<8	<8	8	—	<8	—	—
	7/15	<8	128	<8	<8	32	128	64	16	<8	<8	256	<8	16	32	256	—	64	—	—
KW	6/26	—	8	—	—	—	8	—	16	—	—	32	—	8	<8	16	—	8	—	—
	7/13	<8	32	<8	<8	<8	64	<8	64	<8	<8	128	<8	32	"	128	—	32	—	—
RM	6/22	—	—	—	—	—	—	—	—	—	—	—	—	—	"	<8	—	<8	—	—
	7/13	<8	<8	<8	<8	<8	8	<8	<8	<8	<8	<8	<8	<8	"	"	—	16	—	—
AR	9/13	—	—	—	—	—	—	—	—	—	—	—	—	—	"	—	—	8	—	—
	10/ 1	—	<16	<16	—	<16	<16	<16	<16	—	<16	<16	<16	<16	"	<16	<16	32	<16	<16
WE	6/28	—	—	—	—	—	—	—	—	—	—	<8	—	—	"	—	—	—	—	—
	7/13	—	<16	<16	—	<16	<16	<16	<16	—	<16	"	<16	<16	"	<16	<16	<16	<16	<16
TF	6/22	—	—	—	—	—	—	—	—	—	—	—	—	—	"	—	—	—	—	—
	7/13	—	<16	<16	—	<16	<16	<16	<16	—	<16	<16	<16	<16	"	<16	<16	<16	<16	<16

* — = not tested.

TABLE III. ECHO, Coxsackie, and Poliomyelitis CF Antibody Titers of Sera Collected from 3 Persons before and 28 Days after Experimental Infection with ECHO Type 20 Virus.

Date serum collected	ECHO antigens E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12	E14	E20	Coxsackie B antigens B1	B3	B4	B5	Poliomyelitis antigens P1	P2	P3
7/22/57	<8	<8	64	<8	<8	<8	<8	<8	<8	<8	16	<8	<8	<8	<8	8	8	<8	<8	<8	<8
8/19	"	"	128	"	"	32	"	"	"	"	64	"	"	64	32	32	32	32	"	"	"
7/22	"	"	16	"	<8	"	"	"	"	"	16	"	"	16	<8	16	<8	<8	8	"	"
8/19	"	"	"	"	16	"	"	"	"	"	64	"	"	64	32	64	"	16	<8	"	"
7/22	—*	—	<8	—	—	—	—	—	—	—	—	—	—	<8	<8	<8	"	<8	—	"	"
8/19	—	—	"	—	<8	<8	—	—	—	—	<8	—	—	32	16	32	32	16	—	"	"

* — = not tested.

TABLE IV. ECHO, Coxsackie, and Poliomyelitis CF Antibody Titers of Sera Collected from 5 Persons before and after Experimental Infection with Coxsackie B3 Virus.

Date serum collected	ECHO antigens												Coxsackie B antigens		Poliomyelitis antigens	
	E1	E2	E3	E4	E5	E6	E7	E8	E10	E11	E12	E14	B1	B3	P2	P3
10/ 1/56	—*	—	—	—	—	<8	<8	—	—	—	—	—	<8	<8	—	—
12	<16	<16	<16	<16	<16	64	32	<16	<16	<16	<16	<16	64	256	<16	<16
1	—	—	—	<8	—	—	—	—	—	—	—	—	—	<8	—	—
16	<16	<16	<16	32	<16	<16	<16	<16	<16	<16	<16	<16	<16	128	<16	<16
1	—	—	—	—	—	—	<8	—	—	—	—	—	<8	<8	—	—
20	<16	<16	<16	<16	<16	<16	16	<16	<16	<16	<16	<16	64	128	<16	<16
1	—	—	—	—	—	—	—	—	—	—	—	—	<8	<8	—	—
22	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	"	32	<16	<16
1	—	—	—	—	—	—	—	—	—	—	—	—	"	<8	—	—
12	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	16	<16	<16

* — = not tested.

virus of the enterovirus family might respond only with type-specific CF antibody and that heterologous responses observed in adults were the result of previous enterovirus infections.

Although CF antibody response is of limited usefulness for diagnostic purposes, it does furnish additional evidence of relationships between various members of the enterovirus group. In this study ECHO virus types 2, 4, 5, 6, 7, 8, 11, 14, 16, and 20, and Coxsackie B virus types 1, 3, 4, and 5 were linked to each other by significant heterologous antibody responses. It is theoretically possible that some apparently heterologous responses were due to actual infection with the heterologous virus. However, the short period of time between the 2 serum specimens and age and environment of persons studied make this possibility unlikely.

Although ECHO virus types 1, 3, 9, and 12 were not linked to previously mentioned types by unequivocal CF antibody responses, antigenic relationships between ECHO type 9 and ECHO types 4 and 6, Coxsackie A types 5 and 7, Coxsackie B types 3 and 4, and poliovirus types 1, 2, and 3 have been demonstrated in another investigation(12). Antigenic relationships between poliovirus types and certain other enteroviruses have also been reported in 2 additional studies(13,14). Absence of such reactions in our study may have been the result of small number of sera tested, type of antigen used, technic of the test, dilutions of sera tested, or other unknown factors.

Absence of antigenic relationships between ECHO type 10 virus and the others included in this study is in accord with other data on characteristics of ECHO type 10 virus, which suggest that it is not related to other ECHO viruses(2,15).

Summary. A study of heterologous CF antibody responses in persons naturally or experimentally infected with ECHO virus types 10, 16, and 20, Coxsackie B virus type 3, or poliovirus type 1, indicated an antigenic relationship between the following viruses: ECHO types 2, 4, 5, 6, 7, 8, 11, 14, 16, and 20, Coxsackie B virus types 1, 3, 4, and 5. No antigenic relationship was noted between ECHO

TABLE V. ECHO, Coxsackie, and Poliomyelitis CF Antibody Titers of Sera Collected from Persons in Acute and Convalescent Phase of Type 1 Paralytic Poliomyelitis.

Patient	Date serum collected	ECHO antigens E2	E3	E4	E6	E7	E11	Coxsackie B antigens B1	B3	B5	Poliomyelitis antigens P1	P2	P3
DF	8/ 2/56	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8
	27	"	"	"	"	"	"	"	"	"	32	"	"
RC	9/10	8	8	"	32	16	"	"	"	"	<8	"	"
	10/17	8	8	"	"	"	"	"	"	"	32	"	"
ED	7/25	8	<8	"	<8	<8	"	"	"	"	<8	"	"
	8/13	8	"	"	"	"	"	"	"	"	32	"	"
TG	8/17	8	"	"	16	32	"	"	"	"	<8	"	"
	9/ 7	<8	"	"	8	"	"	"	"	"	16	"	"
DG	8/ 3	"	"	"	<8	<8	"	"	"	"	<8	"	"
	25	"	"	"	"	"	"	"	"	"	16	"	"
RP	9/25	16	8	"	"	16	"	32	32	32	<8	"	"
	10/10	"	8	"	"	"	"	"	"	"	"	"	"

virus type 10 and any of the other enteroviruses.

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Formalin Treated Chicken Erythrocytes as Indicators of Influenza A Virus (Asian) and Its Antibody.* (24897)

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Formalin treated human erythrocytes have been used to measure the hemagglutinating capacity of influenza virus(1,2), detect antibodies against coupled protein(3,4), haptens (4,5) and enterobacterial antigens(6). While such erythrocytes were reported(1) as early as 1948 to be comparable to normal human and

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chicken erythrocytes for adsorbing and eluting influenza A hemagglutinating activity they have not replaced fresh cells perhaps because of the spontaneous agglutination which may occur in the presence of traces of formaldehyde(1,7) or because such cells do not lend themselves well to reading by the usual pattern method(1,3). As there are, however, substantial theoretical advantages to their use, continued efforts have been made toward obtaining preparations that would be suitable for routine hemagglutination and hemagglutination-inhibition tests. Tube titrations of influenza viruses and their antibodies with formalin preserved human erythrocytes recently have been reported by Cox and Pirtle (2) and Ingraham(4) has described the use of treated human erythrocytes for attaching proteins and haptens to detect specific antibodies by means of the tube pattern method. This report will describe the preparation of formalin preserved chicken erythrocytes, their use in titration of the Asian strain of influenza virus A and its antibody and comparison of results obtained in such titrations with preserved and fresh chicken erythrocytes. Similar trials with preserved human and sheep cells proved to be unsatisfactory because of incomplete settling even after overnight incubation.

Material and methods. The diluent used throughout consisted of 0.137 M NaCl in distilled water buffered at pH 7.2 with 0.01 M Na_2HPO_4 and 0.003 M KH_2PO_4 (PBS). Chicken blood was drawn into modified Alsever's solution(8) and stored at 4°C as a 50% suspension which was permitted to stabilize for one week before being used. *Preparation of preserved chicken erythrocytes (PCE).* The procedure described by Cox and Fingerhut(9) as altered by Feeley, Sword and Manclark(6) was followed except for minor modifications. Fifty ml of a 50% suspension of whole blood in Alsever's solution was diluted with an equal volume of PBS. This was rapidly mixed with 40 ml of 50% formalin diluted in twice concentrated PBS. The mixture was incubated for 2 hours in a 37°C water bath. The flask was agitated vigorously every 10 minutes. During incubation, the cells gradually changed in color from red to dark brown. They were then washed 6 times with not less than 40 volumes of PBS. Centrifugation was performed in an International Model V centrifuge (size 2), head #239, at 2,000 rpm for 10 minutes. Since the formaldehyde caused marked clumping of the cells, they were resuspended with a magnetic stirrer for 5 minutes during the washing procedure. Finally, the cells were resuspended in 400 ml of PBS and stored at 4°C for 48 hrs; the supernatant was then decanted, the PBS replaced and the cells resuspended. After a second 48 hour period, the erythrocytes were suspended in PBS to a final concentration of 10% packed cells. To this stock suspension, formaldehyde (final concentration 0.3%) was added and the suspension stored at 4°C until used. These cells were found to retain their original degree of reactivity with A/Asian/influenza virus for at least 3 months.

Before use, however, the cells in the stock suspension required several washings to prevent spontaneous agglutination. Subsequently it was learned that a 10% stock suspension of PCE preserved with merthiolate 1:10,000(3) and stored in small aliquots at 4°C was superior. After 2 months' storage, the cells in such stock suspensions could be adjusted to a final concentration of 0.325% in 0.005% bovine albumin‡ in PBS without additional washing. Both positive and negative patterns were as distinct and the end points as sharp as those observed with PCE in 0.3% formalin. Although we still had to use 0.005% bovine albumin in PBS to prevent occurrence of spontaneous agglutination in the hemagglutination tests, preservation of stock suspensions of stabilized erythrocytes in merthiolate offers a distinct advantage in that it is not necessary to wash the cells before use. *Antigen.* The hemagglutinating antigen used throughout was pooled allantoic fluid, *Influenza A/Asian/Japan/305/57.*§ *Sera* were obtained from medical student volunteers before and 4 and 14 days after receiving 0.1 ml of monovalent 400 CCA Asian/A/Influenza vaccine intradermally (to be published). Each serum was treated with periodate(10) before testing. *Hemagglutination (HA) and hemagglutina-*

‡ Bovine albumin, Fraction I, Armour and Co.

§ We are indebted to Dr. Morris Schaffer, P.H.S. for this antigen.

tion inhibition (HI) tests. The Communicable Disease Center standard technique(10) using 4 units of antigen was employed. Tests were carried out in hemagglutination tubes (Vontes) and read by the pattern method (11), after incubation for one hour at room temperature. Antigen and sera were diluted in PBS. Virus titrations (HA) were performed with 0.5 ml of antigen dilution to which 0.5 ml of either preserved (PCE) or fresh (FCE) chicken erythrocyte suspensions were added. The reciprocal of the initial dilution of virus in the last tube showing complete agglutination was considered to be the end point in the HA tests. Lowest serum dilution measured was 1:10. The HI end point was the reciprocal of the initial dilution of serum in the last tube showing no agglutination (complete inhibition). Before use, the fresh chicken erythrocytes were washed 3 times with PBS and a 0.5% suspension of packed cells made in PBS with or without bovine albumin. Small amounts of the stock 10% suspension of PCE were washed 5 times with at least 10 ml of PBS and final suspensions were prepared either in PBS or serial dilutions of bovine albumin, or in normal rabbit serum inactivated at 56°C for 30 minutes and diluted with PBS.

Results. Optimal concentration of preserved chicken erythrocytes. Spontaneous agglutination and irregular agglutination patterns were observed consistently in the HA tests with PCE when PBS was used to suspend the cells. This was not the case, however, in the HI tests, in which patterns comparable to those obtained with FCE were noted. From this, it was inferred that incorporation of the test sera into the system prevented spontaneous agglutination of the PCE. Therefore, preliminary titrations were performed to test the effects of rabbit serum and bovine albumin on agglutination patterns of PCE in presence of influenza virus. Rabbit serum inactivated at 56°C for 30 minutes was found to inhibit agglutination of both PCE and FCE in the HA tests, when used in either 1%, 0.5% or 0.25% concentration. Spontaneous agglutination of PCE did not occur, however, in the presence of the rabbit serum, the negative patterns being similar to those observed with FCE.

Titrations were performed simultaneously with both FCE and PCE suspended in bovine albumin. A series of 5-fold dilutions of bovine albumin in PBS (0.001 to 0.5%), was used to suspend the PCE in a final concentration of 0.5% by volume, of packed cells. Similar suspensions of FCE were prepared in 0.5%, 0.05% and 0.005% bovine albumin in PBS. A parallel titration of FCE in saline also was performed. Albumin was observed to produce a marked effect on the agglutination patterns of PCE. When the highest dilution (0.001%) was used, both positive and negative patterns were indistinguishable from those in the control titrations of FCE in PBS. Furthermore, numerous, replicate titrations of the virus' hemagglutinating activity with fresh cells (FCE) suspended either in PBS, 0.5%, 0.05% and 0.005% bovine albumin showed no significant differences in their end points. Nonetheless, HA tests with 0.5% suspensions of PCE failed to show distinct end points. When these could be determined, they usually were different from those detected with FCE, the difference frequently being more than one tube. Subsequently, the 0.5% PCE suspensions were found to contain more cells per ml than those of FCE because of shrinkage‖ and to pack more completely during centrifugation. Therefore, it was considered that the differences in end points might be due to different numbers of cells in each suspension. In Table I is summarized an example of an experiment designed to test the validity of this assumption. The variations in end points of PCE agglutination by the Asian strain of influenza virus seem to be related to number of cells in the suspension. The agglutination patterns exhibited by the preserved cells in the 0.325% suspension of PCE in 0.005% bovine albumin were usually found to be in close agreement with those observed with the 0.5% suspensions of FCE. When the cells were suspended in concentrations of 0.3% or less, the tests were unsatisfactory because they were difficult to distinguish from the control tubes. In 27 of 32 hemagglutination ti-

‖ Ratio between arithmetic averages of 8 enumerations of PCE and FCE was 1.33.

TABLE I. Effect of Cell Concentration on Agglutination of Formalin Preserved Chicken Erythrocytes by Asian / A / Influenza Virus.

Erythrocytes			Agglutination‡ dilution								
Kind*	% conc.	Diluent†	50	75	100	150	200	400	800	Control	Titer
FCE	.5	PBS	4	4	4	4	1	0	0	0	150
"	.5	BA	4	4	4	4	0	0	0	0	150
PCE	.5	"	4	3	2	1	1	1	0	0	50
"	.4	"	4	4	2	2	1	1	0	0	75
"	.35	"	4	4	4	3	1	0	0	0	100
"	.325	"	4	4	4	4	0	0	0	0	150
"	.3	"	4	4	4	4	4	4	—	—	—
"	.275	"	—	—	—	—	—	—	—	—	—

* FCE = Fresh chicken erythrocytes. PCE = Preserved chicken erythrocytes.
† PBS = Phosphate buffered saline. BA = 0.005% bovine albumin in PBS.
‡ Agglutination patterns were graded from 0 (no agglutination) to 4 (complete agglutination). — = not readable or not obtained.

trations, performed simultaneously with 0.5% FCE and 0.325% PCE, the end points coincided. Furthermore, when discrepancies did occur, in only one instance did the disagreement amount to more than one tube. The end points of titrations with 0.325% PCE in 0.005% bovine albumin were, as a rule, as easily read as those exhibited by FCE.

Accuracy in replicate titrations of antibody. To determine whether the sensitivity of PCE was comparable to that of FCE when used to titrate Asian/A/influenza antibody, experiments such as that summarized in Table II were performed. Four individuals were selected at random from among 20 volunteers who had received, intradermally, 0.1 ml of 400 CCA monovalent Asian/A/Influenza vaccine (not yet published). From each of these, 3 samples of sera obtained just before (A), 4 days (B) and 14 days (C) after vaccination, were used. Simultaneous titrations with FCE and PCE were performed in paired samples of each serum but the order of titration with each serum was not randomized. These titrations were repeated on 2 different days, using antigen and erythrocytes suspensions from the same pools. The data (Table II) show good agreement between end points when tests were performed with FCE and PCE.

Discussion. That hemagglutination and hemagglutination inhibition procedures are highly sensitive and accurate biological reactions is well established(11,12,13) and supported by the present experience. A major difficulty associated with the HA and HI procedures with freshly prepared erythrocytes, however, is the instability of the red blood cells. Loss of agglutination activity of as much as 50% has been demonstrated after storage of cell suspensions for as short a period as 7 days(12).

Use of formalin preserved cells should be advantageous in reducing the requirement for a constant supply of blood and the necessity for frequent, laborious preparations of cell suspensions. That no detectable loss of hemagglutinating activity of these cells was observed after storage for at least 3 months, is in accordance with other reports(2,4). The high degree of accuracy of replicate titrations, attainable under the conditions described, is comparable whether freshly prepared or formalin preserved cell suspensions were employed. The spontaneous agglutination which is said to occur(1,7) with formalinized cells was observed in the HA tests but not in the HI reactions. Addition of small amounts of bovine albumin to the phosphate buffered saline was sufficient to prevent this difficulty. When the experiments reported herein were nearing completion, a paper by Ingraham(4) on a new method for preparing formalinized human erythrocytes appeared. Cells prepared according to this report are said to be insensitive to traces of formaldehyde. We have prepared chicken and human cells according to this method but the results of titrations of the Asian influenza A virus and its antibody were not superior to those performed with the other cells prepared in the present study. Furthermore, spontaneous agglutination was observed to occur in the HA tests when these cells were suspended in buffered saline. We were, however, dealing with a somewhat dif-

TABLE II. Replicate Hemagglutination-Inhibition Titrations of Asian Influenza A Antibody with Fresh and Formalin Preserved Chicken Erythrocytes in Sera Obtained from Immunized Human Volunteers.

Subject	Serum*	Erythrocytes†	Hemagglutination-inhibition titrations‡ Determination 1	2	3
59-07	A	FCE	10	0§	10
		PCE	"	10	"
	B	FCE	160	160	80
		PCE	"	"	160
	C	FCE	320	640	640
		PCE	"	320	"
60-01	A	FCE	10	0	0
		PCE	"	0	0
	B	FCE	"	0	0
		PCE	"	0	0
	C	FCE	40	40	40
		PCE	"	"	"
60-06	A	FCE	0	0	0
		PCE	0	0	0
	B	FCE	0	10	0
		PCE	0	"	0
	C	FCE	80	40	80
		PCE	"	80	160
60-16	A	FCE	0	0	0
		PCE	0	0	0
	B	FCE	40	40	20
		PCE	"	"	"
	C	FCE	"	"	40
		PCE	"	"	20

* Samples of serum obtained from subjects, before (A); 4 days (B), and 14 days (C) after receiving monovalent Asian influenza vaccine.

† FCE = Fresh chicken erythrocytes. PCE = Preserved chicken erythrocytes.

‡ Reciprocal.

§ 0 = <10.

ferent system. Ingraham(4) had used 1:100 rabbit serum in saline to suspend the cells. That the absence of spontaneous agglutination is related to the rabbit serum is suggested by the present observations that either rabbit serum or small amounts of bovine albumin prevented spontaneous agglutination in the HA tests. The human sera used in the HI reactions have a similar effect. Rabbit serum, however, is not useful in influenza virus titrations, since it often contains non-specific inhibitors(14). When the formalinized cells were adjusted volumetrically to concentrations similar to 0.5% fresh chicken cells, different end points were obtained and the preserved cells usually produced less distinct patterns than fresh cells. The end points of titrations with preserved cells tended to be lower than those employing fresh cells. Since the suspensions of preserved cells were found to contain more cells than the corresponding concentration (by volume) of fresh cells, it appeared as though the lower sensitivity exhibited by the preserved cells was related to the larger number of cells in suspension. If this is the case, then the behavior of cells which have been stabilized by formaldehyde does not differ from fresh cells in this regard either, for it has been observed that with the latter, a simple inverse proportion exists between the highest dilution of the virus preparation causing complete agglutination and the quantity of cells used in the indicator system (11).

Summary. Formalinized chicken erythrocytes were equivalent to fresh cells for measurement of influenza A virus (Asian) and its antibody. The preserved cells were smaller than fresh ones, requiring some volumetric adjustment. It was necessary, also, when titrating HA ability of treated cells to incorporate small amounts of protein into the diluent.

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Effects of ACTH on Plasma Iron Levels in Normal Human Subjects. (24898)

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The mechanism of hypoferremia of infection has been obscure. It has been suggested that adrenal steroids cause a lowering of serum iron levels in dogs and rats(1). Hypoferremia in man after other stresses has also been attributed to adrenal steroid release (2,3). Reports of the effect of adrenocorticotrophic hormone (ACTH) and adrenal steroids on serum iron levels in man have been at variance, and often been equivocal(4-9). The present study compares changes in plasma iron level in normal human subjects, following administration over 8 hours of a standard test dose of ACTH, with changes occurring during comparable control period.

Procedure. Normal healthy volunteers, on regular diet but enjoined from strenuous activity during 3 days of this study, were the subjects. Diurnal-nocturnal variation of their plasma iron concentrations was observed during 24 hour control period. Forty mg of ACTH (Upjohn) in 1000 cc of 5% glucose in water were then administered intravenously for 8 hours. During and following the infusion, plasma iron levels were determined by the method of Schade *et al.*(10). Duplicate determinations were performed on each specimen.

Results. Fig. 1 depicts results when ACTH was administered from 8 a.m. to 4 p.m. when plasma corticosteroid levels normally fall(11). ACTH did not alter plasma iron level, either during or after infusion, that could not be accounted for by diurnal-nocturnal or random variation, as shown by initial 24 hour control period.

Because plasma corticosteroid levels themselves undergo diurnal-nocturnal variation, the experiment was altered so that ACTH was administered when plasma corticosteroid levels normally rise. In Fig. 2, results are shown of administering 40 mg ACTH between 11 p.m. and 7 a.m. after 24 hour control period. Subject K attained quite elevated plasma iron levels during the first 24 hours compared with her later levels. Subject I, on the other hand, reversed this pattern and had a lower plasma iron level during the first 24 hours than on the second day. One may conclude that ACTH did not significantly affect the diurnal-noc-

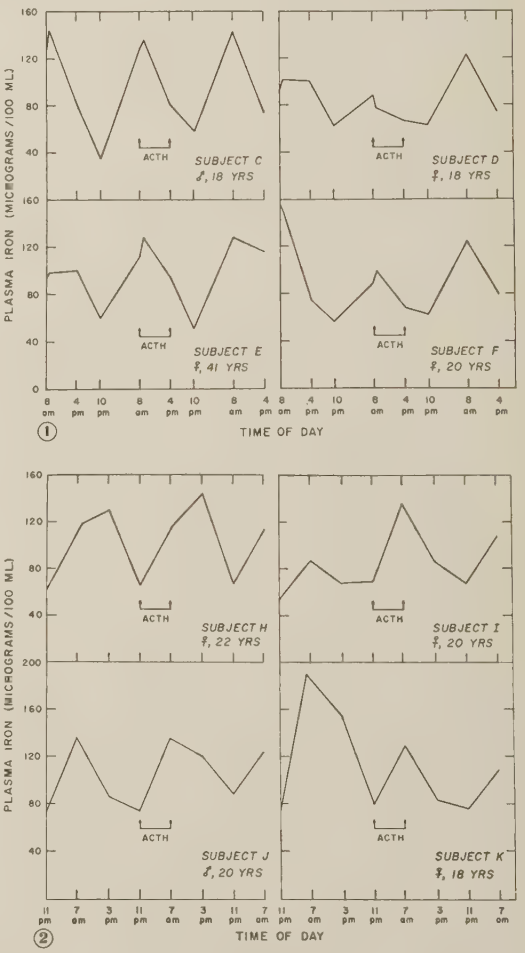

FIG. 1. Changes in plasma iron concentration before and after administration of ACTH. Intrav. infusion, 8 a.m. - 4 p.m.

FIG. 2. Changes in plasma iron concentration before and after administration of ACTH. Intrav. infusion, 11 p.m. - 7 a.m.

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turnal variation of plasma iron in these subjects.

Summary. A standard dose of ACTH was administered intravenously for 8 hours to normal volunteers. Plasma iron levels were not affected in any way that could not be accounted for by normal diurnal-nocturnal or day to day variation. Altering normal daily periodicity of corticosteroid concentration by administering ACTH at different phases of the cycle did not affect the curves of plasma iron variation.

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Tryptophan Oxidation by Yellow Mouse Skin. (24899)

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There has been for some time a good deal of evidence that tryptophan is the source of intermediates in production of pigments in insects (See 1, 2, for reviews), but in mammals, tryptophan has been suggested as a possible precursor of pigments only recently(3). An interesting example was discovered by Foster(4), while studying pigment formation by skins of a strain of mice with yellow fur due to dominant gene, A^y/a. Foster found that skin powders from these mice showed very little ability to oxidize tyrosine, the usual precursor of melanin, but vigorously oxidized tryptophan with production of yellow pigment. While in insects, tryptophan appears to lead to pigments by way of kynurenine(1,2), the pathway recently suggested for mammals and possibly other animals(3), is quite different, namely *via* a 5-hydroxyindole compound. No experiments have been reported to determine which, if either, of these alternative routes may be followed in skins of A^y/a mice. In addition, it is of interest to determine whether or not kynurenine is formed by these skins, since Knox(5) studied a number of tissues from several species, including rat, rabbit, and guinea pig, and reported conversion of tryptophan to kynurenine only in the liver. The experiments reported here indicate that kynurenine is not utilized in pigment formation by skins of A^y/a mice, and that it is probable that a hydroxyindole is an intermediate.

Materials and methods. Mice, obtained from Jackson Memorial Laboratories were A^y/a females, from various matings with pa/pa individuals, and males of a black inbred strain, C57Bl/6. Grüneberg(6) describes the phenotypes. The mice were maintained on stock ration supplemented with occasional fresh vegetables. Only progeny of yellow phenotype were used. Coat color in the A^y/a mice varied from light yellow to deep orange. At 8-11 days after birth, mice were sacrificed by decapitation. Skins from back and head

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TABLE I. Rates of Oxidation of Tryptophan and 5-Hydroxytryptophan by A^y/a Mouse Skin Powders.

Substrate added	Oxygen uptake* (μl/100 min./ml skin)	mg skin/ml (wet wt)		μl O_2/hr/g skin
25 μM L-tryptophan	55	300		110
	69	261		159
	66	277		143
	58	324		105
			Mean	130
12.5 μM "	34	274		73
	37	324		68.5
			Mean	70.7
5 μM "	23	300		46
25 μM DL-5-hydroxytryptophan	44	324		81.5
12.5 μM "	26	274		61
	36	324		57
			Mean	59
25 μM D-tryptophan	−2	261		−7.7

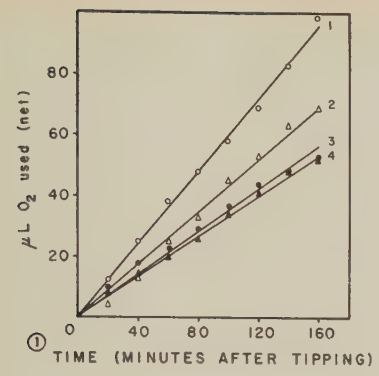
* Total volume 2 ml, conditions as described in text.

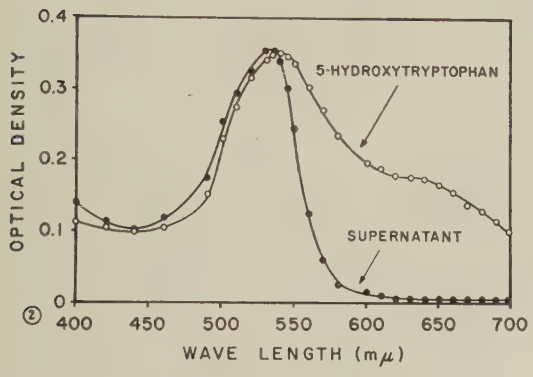
were rapidly removed, weighed on torsion balance, and frozen between blocks of dry ice. Skins from 2-6 mice were pooled. Skin powders were prepared by grinding in mortar as described by Foster(4). They were either used at once or after storage at −20°C for 1-5 days. Measurements of oxygen uptake were made in Warburg vessels(7) in an atmosphere of oxygen at 37.3°C. Routinely, 1 or 1.5 ml of skin powder suspension made up in sodium phosphate buffer, pH 6.8, or 6.0, 0.2 molar, was placed in main compartment, with 0.5 ml of substrate in buffer or buffer alone in the side arm, and 0.2 ml of 20% KOH in center well. Phosphate buffer was used to bring contents to a total of 2 or 3 ml. All substrates were purchased from California Fn. for Biochemical Research. Substrate concentrations in sidearm varied from 10 to 50 μM/ml of L-tryptophan, DL-5-hydroxytryptophan, and 5-hydroxytryptamine; L-kynurenine and D-tryptophan were 25 and 50 μM/ml. After incubation, skin particles were removed by centrifugation and 1 ml of supernatant solution was deproteinized with 1 ml of 0.3 molar zinc sulfate, or total reaction mixture was added to 2 ml 0.3 M zinc sulfate. This precipitant permitted assay of kynurenine formed by liver homogenate from tryptophan as readily as by Knox's method(5), and requires less dilution. Measurements of optical density were made in Beckmann model DU spectrophotometer with cells of 1 cm light path. These measurements were made, with appropriate blanks, both on supernatants after treatment with zinc sulfate and on an aliquot of supernatant assayed for 5-hydroxyindole by the nitrosonaphthol method of Udenfriend *et al.*(8), run at 40°C.† Spectrum of the chromophore produced was compared with that of authentic 5-hydroxytryptophan or 5-hydroxytryptamine added to a skin suspension blank just before centrifugation. Chromophores were extracted into equal volumes of n-butanol, and spectra were again recorded.

Results. Initial experiments showed that skins from 10-day-old mice oxidized L- but not D-tryptophan (Table I). This is in agreement with Foster's observation that L-tryptophan was oxidized more rapidly than DL-tryptophan(4). Rates of oxidation depend on concentration of L-tryptophan over the range studied (Table I). The resultant supernatants directly after incubation were red, but on standing at room temperature changed to yellow.

The following experiments were carried out to determine whether or not kynurenine might be an intermediate. First, after incubation

† Tryptophan contributes to absorption when more than 4 or 5 μM are present in the assay, but does not change the peak or shape of curves. Since its final concentration is not known no correction has been made for it in Fig. 2.





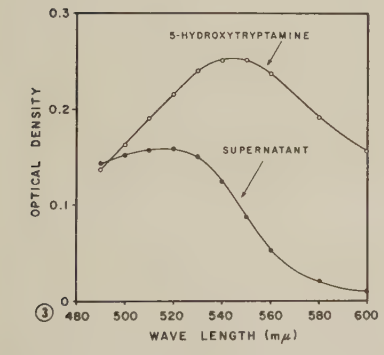


FIG. 1. Oxygen uptake by skin suspensions in presence of : Curve 1, 25 μM L-tryptophan; curve 2, 25 μM DL-5-hydroxytryptophan; curve 3, 12.5 μM L-tryptophan; curve 4, 12.5 μM DL-5-hydroxytryptophan.

FIG. 2. Absorption spectra of chromophores produced with nitrosonaphthol by supernatant after incubation with 25 μM L-tryptophan and by authentic 5-hydroxytryptophan (0.2 μM).

FIG. 3. Absorption spectra of chromophores of supernatant and of 5-hydroxytryptamine after extraction into n-butanol.

with tryptophan and precipitation with zinc sulfate, absorption spectra of supernatant solutions were examined. No peak was observed at 365 mμ, corresponding to absorption maximum for kynurenine. Secondly, L-kynurenine was incubated with skins for 2 and 3 hours to see whether oxygen uptake would result, as pigment formation would be expected to require further oxidation. No net oxygen uptake over controls was observed, nor was any additional color produced in the supernatant. Thirdly, disappearance of kynurenine was studied. Kynurenine was incubated with skins for 2 and 3 hours. Optical density at 365 mμ of diluted aliquots of supernatants was then compared with that from vessels containing buffer alone plus an equivalent amount of kynurenine. These optical densities were the same, within a 5% error of measurement.

Since 5-hydroxytryptamine, another metabolite of tryptophan, may give rise to pigments when incubated with various tissues(9, 10,3), its precursor, 5-hydroxytryptophan, was incubated with skin suspensions. It was found that with 2 concentrations of DL-5-hydroxytryptophan oxygen was taken up nearly as rapidly as with equimolar concentrations of L-tryptophan (Fig. 2, Table I). 5-hydroxytryptophan gave rise in all cases to solutions which were yellow in color both initially and on standing. 5-hydroxytryptamine was also oxidized, yielding paler solutions.

The nitrosonaphthol test for 5-hydroxyindoles was performed on supernatants after incubation with tryptophan, and a distinctive chromophore resulted, the yield being greater after incubation at pH 6.0 than pH 6.8. However, it was not violet in color, as is that formed by 5-hydroxyindoles, but red. The absorption spectrum was compared with that of the chromophore due to 5-hydroxyindoles added to skin blanks before centrifugation. A slight but definite shift in peak absorption was observed and a marked difference in optical density at wave lengths above 550 mμ (Fig. 3). The chromophore due to 5-hydroxyindole was not changed above 500 mμ by presence of excess tryptophan, nor was it altered by presence or absence of skin suspension prior to precipitation. Differences between chromophores produced by the supernatant after incubation and by 5-hydroxyindoles persisted when chromophores were extracted into n-butanol (Fig. 3).

Discussion. These results indicate that kynurenine does not appear to be an intermediate in pigment formation by skins of A^y/a mice. The fact that DL-5-hydroxytryptophan is oxidized nearly as rapidly as equimolar L-tryptophan means that L-5-hydroxytryptophan is probably oxidized more rapidly than L-tryptophan. This suggests that the hydroxylated form may be an intermediate in formation of the colored substance. In the absence of strong nitric acid, the nitrosonaphthol reagent is said to be quite specific for 5-hydroxyindoles when the violet chromophore results, and a number of compounds have been examined(8). Since supernatants after incubation with tryptophan yield a substance forming a similar but not identical chromophore, it is possible that a related hydroxyindole or a di-hydroxyindole is formed. It is unlikely that the difference in the chromophore is due to an impurity, since 5-hydroxytryptophan was added to skin suspensions before taking the spectra, and the chromophore produced was identical with that from 5-hydroxytryptophan alone. In addition, it was found that the chromophore of 5-hydroxytryptophan was not altered by excess tryptophan, and finally, an impurity might be expected to add, rather than subtract, optical density. Further experiments will be required to determine whether the colored substance formed may be due to action of an amine oxidase on a hydroxytryptamine, as has been described(9,10), or whether a di-hydroxyindole may lead to a quinone, as has been suggested(3).

Summary. 1. Skin powders from 10-day-old mice with a dominant gene for yellow hair (A^y/a) oxidize L-tryptophan, DL-5-hydroxytryptophan, and 5-hydroxytryptamine, but not D-tryptophan. Yellow solutions are formed. 2. L-kynurenine does not appear to be an intermediate, since it is not oxidized, nor does it disappear at an appreciable rate when incubated with skin powders from these mice. It does not appear when tryptophan is incubated with skin powders. 3. When tryptophan is oxidized by these skin powders, a substance appears which gives a reaction with nitrosonaphthol in the absence of strong nitric acid. The chromophore is distinctive, and differs from that formed by 5-hydroxyindoles.

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Differential Action of Styramate and Meprobamate on Spinal Reflexes. (24900)

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Styramate, (Sinaxar®) 2 phenyl-2 hydroxyethyl carbamate, a drug with central depressing action has been recently recommended by us as skeletal muscle relaxant for human use. Its pharmacological, toxicological and anticonvulsive properties have been described(1) and preliminary data on its skeletal muscle relaxant activity have been presented(2). In general its properties are similar to those of mephenesin except that it is 3 to 6 times longer acting. The influence of styramate on spinal polysynaptic transmission is not asso-

ciated with hypnosis as with pentobarbital. The present report contains findings concerning its effects on polysynaptic reflexes in spinal and in decerebrate cats. In our investigation augmentation of patellar reflex during contralateral sciatic nerve stimulation (SCF), which gives results parallel to electrophysiological determinations(3-5), was selected as method of measure.

Method. Forty-one cats (18 decerebrate and 23 spinal) were anesthetized with ether and tracheotomized. Decerebration was made by sectioning brainstem rostral to superior colliculi. Spinal animals were prepared by transecting the cord at C_1. Respiration was maintained in decerebrate and spinal animals by cut-off valve respiratory pump (18/min at 10 lb/sq in). Pharmacological studies were performed 3 hours after ether anesthesia. Patellar reflex was elicited by mechanically stimulating patellar tendon with percussion hammer driven by 6/min counterclockwise motor. Bipolar platinum shielded electrodes attached to central stump of sectioned contralateral sciatic nerve were used. Stimulation (120 cps, 0.5 msec. and 0.1 to 1.0 V) was induced with Thomas square wave stimulator. Selection of frequency was based upon previous work(6) which showed that low frequencies (1 and 10/sec) produced inhibition, and high frequencies (60 and 120/sec) augmentation of patellar reflex. This augmentation was expressed as percent increase in height of knee jerk response during contralateral sciatic nerve stimulation in comparison to prestimulation response. Voltage required varied from animal to animal, but for each animal it was constant except after drug treatment. For greater constancy, the drug effect was determined by establishing minimal dose necessary to completely contain the patellar reflex response at prestimulation values with intensity of stimulation inducing 100% augmentation. Intravenous injection of small multiple doses (1 to 10 mg/kg) in saline for 30 minutes were used to find proper dose range. Afterwards, single doses were used for accurate measurements.

Results. Styramate in 9 high cervical spinal cats abolished SCF in doses of 15 to 40 mg/kg, average of 21.1 mg/kg. In no instance did a dose lower than 15 mg/kg produce complete depression of SCF. On the other hand, in 5 decerebrate cats, the dose required for same effect was less than 5 mg/kg (avg 2.8 mg/kg) whereas in 3 other cats the doses were 10 to 20 mg/kg (avg 13.3 mg/kg). Thus, total average for the 8 cats was 5.3 mg/kg. To establish whether or not the greater susceptibility of the decerebrate animal to styramate is due to nature of animal preparation, we tested other polysynaptic blocking agents. Meprobamate in 4 spinal cats abolished the SCF at doses of 15 to 40 mg/kg with average of 35 mg/kg. In 6 decerebrate cats, meprobamate was effective in blocking SCF at similar dose; namely, 20 to 40 mg/kg, average of 27.5 mg/kg. Phenaglycadol and chlorpromazine tested on 14 cats showed that their action on the decerebrate animal is greater than on the spinal. Since population distribution of minimal (SCF) dose of styramate in decerebrate cats tended to the left, it may be inferred that the average dose of 5.3 mg/kg represents an upper limit. This makes comparison with average dose of 21.1 mg/kg, required in spinal animal in which the distribution was normal, much more pronounced. In contrast, meprobamate appeared approximately equal in effectiveness in decerebrate and spinal cats.

Height of patellar reflex response *per se* in spinal cats was increased by styramate in doses of 10 mg/kg or less and not modified further by doses sufficient to abolish SCF. Meprobamate, at dose levels required to abolish SCF, did not affect the patellar reflex in spinal cats. In contrast, both styramate and meprobamate in decerebrate cats did produce a transient decrease in height of patellar reflex response when doses needed to abolish SCF were employed. This effect on patellar reflex lasted only a few minutes (1 to 5), whereas the effect on SCF persisted 30 or more minutes.

Discussion. Two drugs, styramate and meprobamate, with central skeletal muscle relaxant properties appear to exert similar pharmacological effects on spinal polysynaptic reflex (SCF) in high cervical spinal cats. In decerebrate cats, styramate is about 4 times more potent than in spinal cats. In contrast,

meprobamate does not exhibit this difference in activity in the 2 animal preparations. This suggests that at the spinal level, both drugs exert similar net effects but at supraspinal levels their actions are different. Mephenesin, another relaxant drug, does act on supraspinal reflex transmissions(7) particularly the reticular facilitatory centers influencing spinal reflexes(8). It is likely that styramate which showed other similarities with mephenesin, may affect such areas. A generalization of our conclusions would lead to the possibility that centrally acting skeletal muscle relaxants may independently affect reticular facilitatory centers without influencing the ascending arousal system. Styramate and mephenesin are examples of this type of drug. On the other hand, there are relaxants *e.g.* pentobarbital, which at same dose level, depress both facilitatory and arousal systems. Meprobamate, another relaxant drug, does not affect the facilitatory system, assuming this is the correct interpretation of our data. Its action on arousal is controversial(9,10) but whether it depresses arousal or not, it still appears to belong to a class different from styramate and mephenesin. At this time, it can only be speculated whether differences encountered in our investigation between styramate and meprobamate are in some way related to their differences in anticonvulsant activity. Styramate in mice is much more effective in preventing extensor tonus spasm of hind leg following electroshock, Metrazol and strychnine induced seizures than meprobamate(1).

Summary. Effects of styramate and meprobamate upon spinal polysynaptic (SCF) and monosynaptic (patellar) reflexes in decerebrate and spinal cats were investigated. Both drugs selectively depressed the spinal polysynaptic reflex, however styramate was much more potent in decerebrate than in spinal cats. The significance of this selective effect was discussed in relation to other actions of muscle relaxants.

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Intracerebral Passage of Sarcoma 180 in Mice and Hamsters.* (24901)

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A number of investigators have transplanted neoplasms, including those of human origin, into brains of laboratory animals. Early literature has been reviewed by Sailer (1) and Vazquez-Lopez(2). Recently other investigators(3-7) have used intracerebral (IC) route for biologic studies of various experimental neoplasms. Sarcoma 180 is an easily transplantable murine tumor, growing well in any strain of mice(8) and has also been adapted to growth in tissue culture (TC) (9). However, despite these facts it has not been previously passed intracerebrally. It seemed probable therefore that

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IC passage of this tumor might offer a means of uncovering its variants and of altering its host range. The present report describes serial IC passage of sarcoma 180 in mice and subsequently in suckling hamsters. A TC strain of sarcoma 180 cells was also inoculated into mice for comparative study.

Materials and methods. Sarcoma. Sarcoma 180 was from 2 sources: (a) A subcutaneous tumor in DBA mouse kindly supplied by Dr. Margaret Kelly of Nat. Cancer Inst., and (b) A tissue culture of tumor cells (in 18th tissue culture passage) supplied by Dr. Harry Eagle of Nat. Inst. of Allergy and Infectious Diseases. Suckling Swiss mice, less than 1 day old, and adult Swiss mice, 3-4 weeks old, were from NIH randomly bred or inbred stock. Hamsters (*Mesocricetus auratus*) usually less than 1 day old, were used. All animals were observed for 4 weeks after inoculation. *Tissue cultures.* Passages of sarcoma cells were made at weekly intervals in 32 oz. Blake bottles, using Eagle's medium with 10% horse serum. Cells were scraped off the glass, centrifuged 350 rpm 15 minutes, and transferred to fresh bottles. For animal inoculation, cells were suspended in Eagle's medium, with 2% calf serum. For cell counts, 0.5 ml of this suspension was mixed with 1 ml of 0.1% crystal violet in 0.1 M citric acid. *Passage in animals.* Tumor or brain tissue was minced with scissors and 20% suspensions by weight were made in Eagle's medium with 2% calf serum. After about 5 minutes, the supernate was inoculated into animals. For titration, the supernate of a 10% suspension usually containing about 5 x 10^6 sarcoma cells/ml was used. These cells were identifiable by their large size and dark staining characteristics (Giemsa). *Histology.* Impression smears of brains were stained by the Giemsa method. Tissues fixed in 10% buffered formalin were sectioned and stained with hematoxylin-eosin.

Results. Mice. Sarcoma 180 from DBA mouse was passed intracerebrally (IC) to an adult C_3H mouse. The brain tissue from this mouse was used to establish 2 passage lines: (a) IC in suckling Swiss mice. (b) IC in adult Swiss mice. A third passage line was initiated directly from the DBA mouse and carried in adult Swiss mice by subcutaneous (SC) route. These have been maintained for 30, 25, and 12 passages respectively.

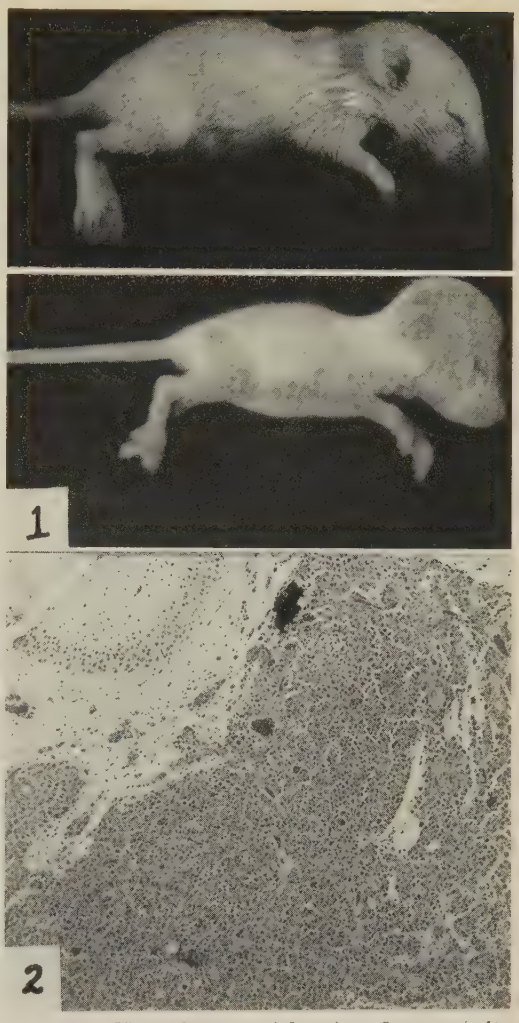

FIG. 1. Normal mouse (above) and mouse with brain sarcoma (below) showing enlargement of head.

FIG. 2. Section of brain of a suckling mouse inoculated IC with sarcoma 180 and sacrificed on 7th day after inoculation, showing sarcoma cells filling lateral ventricle and invading cerebral tissue. Hematoxylin and eosin, ×73.

Suckling mice inoculated IC showed swelling of head in 5 to 6 days (Fig. 1), and failed to gain weight normally. Occasionally, a tumor protruded through the skull. Death usually occurred in 9-11 days. Adult mice inoculated IC did not show cranial enlargement but occasionally developed leg paralysis; they usually died in 9-13 days. All IC passage

TABLE I. Results of Injection of a Subcutaneous Sarcoma Suspension into Adult and Suckling Mice by Different Routes.

Source of suspension	Inoculum (ml)	Route	Mice		No. with tumors	No. died	Avg time (days) until death
			Age	No.			
From 7th subcut. passage	.05	Subcut.	Adult	30	4	0	
	.01	Intracer.	Suckling	10	10	10	12.6
	"	Intraper.	"	11	10	10	20.3
	"	Subcut.	"	12	4	1	17
From 8th subcut. passage	.1	Subcut.	Adult	30	22	3	26
	.01	Intracer.	Suckling	10	10	10	13
	"	Intraper.	"	10	10	10	13.7
	"	Subcut.	"	10	9	4	17.5

mice, suckling and adult, inoculated with the 20% suspension, died.

Brains of mice inoculated IC were unusually soft, with whitish surface areas. Impression smears of cut surface of brain showed numerous round or oval cells with blue cytoplasm and large purple nuclei. Some cells were in bundles. There was great variation in size and shape. These cells, not found in uninoculated mice, were considered to be from the sarcoma.

All mice of the SC passage series developed tumors at site of injection but only 25% of these mice died during period of 4 weeks. In some mice the tumor regressed and consequently the mice recovered. The 20% brain suspensions from the IC series in suckling mice also caused tumors in all mice by the SC route.

Suspensions of tumor tissue from 7th and 8th subcutaneous passages were inoculated into suckling mice by IC, SC, and intraperitoneal (IP) routes. Results (Table I) indicated that the IC and IP injection of suckling mice caused rapidly fatal tumors, and that suckling mice were more sensitive than adults when injected SC.

Brain suspensions from 22nd, 26th, and 31st IC passages in suckling mice produced tumors in all adult mice inoculated SC (0.1 ml) at a dilution of 10^{-1} (Table II) but did not give 100% "takes" at higher dilutions. The same suspensions given IC to suckling mice (0.01 ml) killed all mice at dilutions as high as 10^{-3}, and once at 10^{-4} (Table II). The IC injection of suckling mice is thus about 1,000 times more sensitive for detecting tumor cells than SC injection of adults.

Hamsters. A brain suspension from 16th IC passage of suckling mice was inoculated intracerebrally into suckling hamsters, less than 1 day old. A number developed neurologic signs including paralysis of legs. Some died in 2 or 3 weeks while others recovered. Second passage was not successful. However, after 6 alternate IC passages in suckling mice and hamsters, the sarcoma cells became adapted to continuous IC passage in hamsters, killing more animals in a shorter time. To date, it is in its 12th consecutive hamster passage, in which all inoculated hamsters succumbed except a few which were killed. Inoculation of brain suspensions of these hamsters caused sarcoma in mice.

In earlier IC passages, some hamsters developed swollen heads in second or third week when the animals were already of good size. Some of these were sacrificed and at autopsy a large cavity filled with bloody fluid was found in the brain which was reduced to less than one-half its normal size. Sarcoma cells were found in brain tissue and bloody fluid.

Histopathology. In IC inoculated adult mice there was proliferation of undifferentiated cells which completely filled the meningeal spaces. Proliferation of tumor cells occurred around and in the arachnoid membrane, and around penetrating blood vessels, but no invasion of brain tissue was seen. Similar lesions were found in IC inoculated suckling mice but proliferation of tumor cells was much greater and there was invasion of brain tissue (Fig. 2). In 2 suckling mice there was almost complete replacement of brain by tumor tissue. There were areas of destruction and hemorrhage in the remaining brain tissue surrounding tumor masses. In IP inoculated suckling mice there were large tumor masses

TABLE II. Titrations of Sarcoma 180 (Brain Suspension) in Suckling and Adult Mice by Different Routes.

Source of brain suspension	Dilution	Inoculum (ml)	Route	Mice		No. with tumors	No. died	Avg time (days) until death
				Age	No.			
22nd intracer. passage in suckling mice	10^{-1}	.1	Subcut.	Adult	30	30	11	21.1
	10^{-2}	"	"		30	24	5	22.2
	10^{-3}	"	"		30	6	0	
26th intracer. passage in suckling mice	10^{-1}	.01	Intracer.	Suckling	11	*	11	11
	10^{-2}	"	"		7	"	7	12
	10^{-3}	"	"		8	"	8	14.5
	10^{-4}	"	"		11	"	8	16.4
	10^{-5}	"	"		11	"	2	20
31st intracer. passage in suckling mice	10^{-1}	.1	Subcut.	Adult	20	20	4	26
	10^{-2}	"	"		20	16	0	
	10^{-3}	"	"		0	0	0	
	10^{-1}	.01	Intracer.	Suckling	9	*	9	9.3
	10^{-2}	"	"		9	"	9	10.6
	10^{-3}	"	"		9	"	9	11.4
	10^{-4}	"	"		9	"	9	15.3
	10^{-5}	"	"		9	"	7	16.3
22nd intracer. passage in adult mice	10^{-1}	.03	Intracer.	Adult	10	"	10	11
	10^{-2}	"	"		10	"	10	14.3
	10^{-3}	"	"		10	"	10	15.5
	10^{-4}	"	"		10	"	9	16.6
	10^{-5}	"	"		10	"	0	

* Those mice which died after intracer. inoculation all developed swollen heads.

throughout the abdomen, surrounding the liver, spleen, adrenals and gastrointestinal tract. There was invasion of liver tissue and lungs on one occasion with many small tumor nodules throughout each organ. In some young hamsters, tumor cells appeared to have a predilection for the lateral ventricles. They proliferated there, undergoing necrosis and causing dilatation of ventricles. The proliferation of cells seemed limited, necrosis occurred and the end result was hydrocephalus.

Inoculation of tissue culture sarcoma 180 cells into mice. The strain of TC sarcoma cells was inoculated at its various passage levels into suckling mice by the IC route and into adult mice by the subcutaneous route. A typical titration experiment is represented in Table III. About 500,000 sarcoma cells were required to give 100% "take" by subcutaneous route in adult mice. This finding is in agreement with that of Zahl(10) who used cell suspensions made from subcutaneous tumors. After IC inoculation of suckling mice with comparable doses, the TC sarcoma cells behaved differently from those of mouse origin. The death rate was much lower and those mice which eventually died survived much longer when TC sarcoma cells were used than when brain tumor tissue was used. Protruding tumors on head were often found when TC sarcoma cells were inoculated IC in suckling mice. In TC, sarcoma cells were of

TABLE III. Titration of 25th T. C. Passage of Sarcoma 180 Cells in Adult and Suckling Mice by Different Routes.

Inoculum (No. of cells)	Route	Mice		No. with tumors	No. died	Avg time (days) until death
		Age	No.			
500,000	Subcut.	Adult	10	10	0	
50,000			10	4	0	
5,000			10	0	0	
50,000	Intracer.	Suckling	11	11	6	20.5
5,000			11	11	0	
500			11	7	2	17.5
50			10	6	0	
5			9	4	1	18

2 types, rounded or spindle-shaped as observed previously by Abercrombie *et al.*(9). This was in contrast to impression smears from brains of mice or hamsters inoculated with either TC or animal passage sarcoma, where only the rounded forms were found.

Discussion. This study, in which suspensions of tumor cells were injected IC with syringe and needle (as distinguished from earlier use of a trocar) gave clear cut results. About 5 x 10^5 sarcoma cells from mouse brain gave 100% "take" by subcutaneous route in adult mice, but only 1/1,000 of that quantity caused 100% mortality by IC route in suckling mice.

Although sarcoma 180 is traditionally regarded as strictly species specific(8), in our study it has been adapted to hamsters, and more than 12 consecutive passages have been carried out in these animals. In early passages some hamsters developed hydrocephalus. According to Murphy and Sturm(11) positive results of heterologous transplantation were due to absence of a lymphocytic defensive reaction in the nervous system, but tumors were not produced when the implanted fragment touched the ventricular surfaces, which reacted like subcutaneous tissue. The question of the exact mechanism underlying development of hydrocephalus in hamsters cannot be answered at present. Further studies are now in progress.

Sarcoma cells after more than 25 passages in tissue culture produced sarcoma in adult mice regularly after subcutaneous injection. Suckling mice inoculated with TC sarcoma cells by the IC route showed a much lower mortality rate and longer survival time than those inoculated with sarcoma cells from mouse brain. Whether these 2 lines of sarcoma cells are true variants or not remains to be determined.

Summary. Mouse sarcoma 180 has been through a total of more than 25 IC passages in suckling and adult mice. Sarcomatous brain suspensions also produced sarcomas in adult mice by subcutaneous route. The IC route in suckling mice was about 1,000 times as effective as subcutaneous route in adult mice. After serial IC passages in suckling mice the sarcoma was adapted to suckling hamsters by IC injection and to date more than 12 serial passages have been carried out in this animal. A strain of sarcoma 180 cells which has undergone more than 25 TC passages produced sarcomas regularly in adult mice after subcutaneous injection and required about 500,000 cells to give 100% "takes." However, when inoculated IC into suckling mice these cells seemed less neurotropic than mouse (IC) passage sarcoma cells.

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Arthropod-Borne Virus Plaques in Agar Overlaid Tube Cultures.*† (24902)

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The agar overlay technic as originally described by Dulbecco(1) and modified by Hsiung and Melnick(2), has been adapted for use in studying plaque formation of selected arthropod-borne (arbor) viruses in tube tissue cultures. It has become evident in working with this group that virus-induced cytopathogenic effects (CPE) in fluid cultures and plaque formation in parallel agar overlaid cultures did not always coincide. In a number of instances it was found that in similar tissue culture systems, virus multiplication proceeded in fluid cultures without evidence of CPE, but it could be detected by formation of plaques under an agar overlay. With use of tube overlay technic, it has thus been possible in many instances to detect multiplication of certain arbor viruses by formation of plaques when cytopathic reactions in fluid cultures were doubtful or not evident. This method may, therefore, be useful for recovering many members of this group of viruses which would have otherwise been missed if CPE were used as the only criterion for tissue culture susceptibility to virus multiplication. The method also lends itself quite satisfactorily to the performance of serum neutralization tests.

Methods. Tube cultures (16 x 100 mm test tubes) were prepared from freshly trypsinized Pekin duck and rhesus monkey kidney and chick embryo tissues in the usual manner(3, 4). Growth medium for all cultures consisted of 0.5% lactalbumin hydrolysate and 2% calf serum in Hanks' balanced salt solution(5). Earle's medium containing 2% calf serum was used to maintain cell monolayers once they had become confluent. Virus log or half log dilutions in Earle's medium were freshly prepared and inoculated in 0.2 ml amounts into pre-drained tube cultures. All tissue culture tubes were incubated 2 hours at 33-35°C to allow virus adsorption to the cell monolayer. After washing, each cell sheet was then covered with 2 ml of the agar overlay medium (2) which contained a final concentration of 2% agar and 2% calf serum. The tubes remained in slanted position during delivery of overlay. After the agar had solidified, the tubes were stoppered and incubated 2-8 days or until plaques became evident. Following initial incubation period of 12-14 hours, it was best to invert the tubes so that any condensed moisture could drain from the agar slants.

Results. Differentiation of arbor viruses within sero-groups A, B and C by plaque size or shape was extremely difficult or not possible. However, differences between these groups could be shown by plaque formation in specific tissue culture systems (Table I). Formation of plaques following virus seeding generally required 2-3 days for viruses of sero-group A in chick embryo, 5-6 days for group B in Pekin duck kidney, and 6-7 days for group C in rhesus monkey kidney tissue cultures. The period required for CPE, when it occurred, was usually the same as required for appearance of plaques.

The type of virus titration endpoint obtained by the plaque method in tube cultures of chick embryo is illustrated in Fig. 1. The plaques result in cells losing the neutral red stain and appear as white transparent colonies upon a pink background. As will be observed, number of plaques relates to dilution of the virus. The 10^{-6} dilution of Eastern equine encephalomyelitis (EEE) virus titration with normal mouse serum (upper figure) shows no isolated plaques as the plaques have become confluent and completely decolorized the cell layer. Whereas, distinct plaques appear as the endpoint of titration in the 10^{-10} dilution. Plaque-forming unit (PFU) deter-

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TABLE I. Comparative Titration of Representative Arthropod-Borne Viruses.

Sero-group	Virus strain	No. mouse pass.	Tissue culture system	Tube TC titer/0.1 ml		Mouse
				TCD_{50}	PFU*	LD_{50}/.02 ml
				Log		
A	EEE RF B-53677	3	Chick embryo	7.1	8.5	7.4
	Middleburg RF 254557	13		<2.0	7.4	6.0
B	West Nile Ar 248	11	Pekin duck kidney	6.2	7.0	6.4
	St. Louis RF B-50487	122		<2.0	5.8	5.1
C	Marituba† Be An 15	16	Rhesus monkey kidney	<2.0	6.6	6.0
	Oribocat† Be An 17	4		<2.0	7.0	6.5

* Plaque-forming units.

† Isolated by O. R. and C. E. Causey, reported by Theiler, M. and Casals, J., *Klin. Wochensch.*, 1959, v37, 59.

minations were found, in most instances, to yield higher titers than TCD_{50} (when CPE occurred) or infant mouse LD_{50} titers (Table I).

Fig. 1 also illustrates the application of this method in a serum neutralization test. Heat inactivated normal and EEE immune sera, each at a 1-20 dilution, were mixed with equal amount of increasing log dilutions of the virus. After one hour incubation at room temperature, the mixtures were inoculated into prepared tissue culture tubes. It will be noted that with the normal mouse serum-virus mixture, the tube containing 10^{-10} dilution of the virus shows plaques, while in immune mouse serum-virus mixtures, the highest dilution of virus to show plaques is at 10^{-6}. This indicates that approximately 4-5 logs of virus were neutralized by the immune serum. It was customary to run 3 tubes for each virus dilution, and average plaque endpoint was recorded as the product of average number of plaques formed and corresponding virus dilution.

Summary and conclusions. A tissue culture tube overlay method for detecting virus growth and for performing titrations and serum neutralization tests with members of the arthropod-borne viruses has been described. This method was more consistent and more sensitive than cytopathogenic effects for detecting multiplication of certain

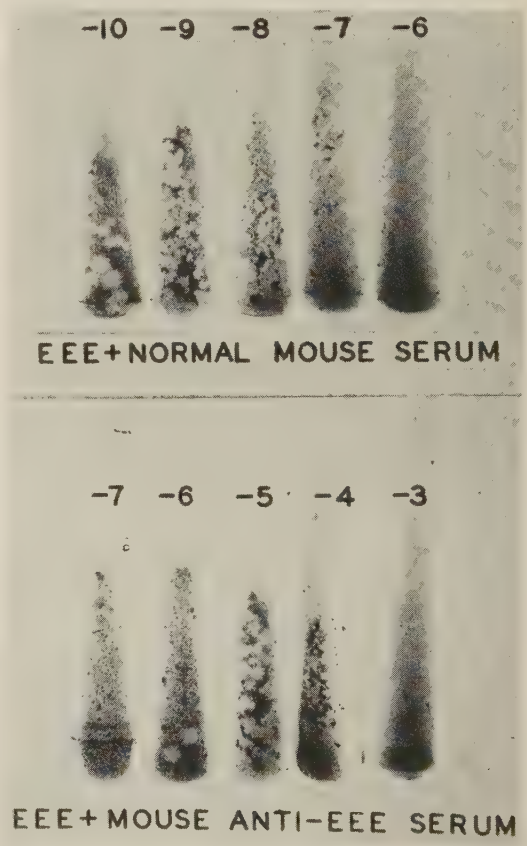

FIG. 1. Serum neutralization test of eastern equine encephalomyelitis virus in chick embryo tube cultures. Upper: Increasing virus $\log_{10}$ dilutions mixed with heat inactivated normal mouse serum, 1-20 dilution. Lower: Increasing virus $\log_{10}$ dilutions mixed with heat inactivated anti-EEE mouse serum, 1-20 dilution.

arbor viruses in tissue culture. Plaque-forming unit endpoint titrations were generally higher than the cytopathogenic endpoint (TCD_{50}) even when CPE was evident, or the endpoint (LD_{50}) in infant mice inoculated intracerebrally. It is suggested that the tube overlay technic may be an aid in large scale screening of clinical or field specimens and in conducting serum neutralizing antibody surveys for many arthropod-borne viruses.

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Investigation of Rejection of Canine Renal Homotransplants by Fluorescent Antibody Technic.* (24903)

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It is widely held that rejection of transplants of homologous tissue results from an acquired immunity expressed by host to donor tissue. However, identification and classification of this type of immune process has remained enigmatic. Attempts to relate circulating antibody with homograft rejection have been conflicting(1,2,3). No changes in serum complement(3) or properidin(4) have been found. It appeared worthwhile to study renal homotransplants in various phases of rejection for presence of recipient's globulin by fluorescent antibody technic of Coons and Kaplan(5). Utilization of so-called indirect modification of the latter also allowed for possible detection of circulating antibodies in recipient's serum.

Materials and methods. Renal homotransplantation was performed on adult mongrel dogs by uniform surgical procedure used in renal homotransplant investigations. The right kidney and a segment of ureter of donor was severed with a cuff of aorta and vena cava attached to renal artery and vein respectively. After removal of recipient's right kidney, anastomosis of the vascular cuff with recipient's aorta and vena cava just above trifurcation was performed using standard vascular anastomotic technics. The ureter was implanted into recipient's urinary bladder. Left kidney of recipient was removed either simultaneously or 24 hours later through separate flank incision. Renal homotransplants were obtained by sacrificing 10 dogs prior to exhibition of rejection as indicated by normal BUN, 8 in an early phase of rejection (moderately elevated BUN) and 8 with moderate to marked elevation of BUN, often of several days duration. Portions of transplanted kidneys and their renal arteries, as well as from untreated controls were quickly frozen on dry ice. Sections were cut at 5 μ in a cryostat and stained with antisera containing either anti-dog globulin (anti-DG), anti-dog albumen (anti-DSA), or anti-dog fibrinogen (anti-DF) which had been conjugated with fluorescein isocyanate. Antisera were obtained by immunizing rabbits with fractionated canine plasma proteins prepared by continuous flow electrophoresis. Technical controls consisted of sections similarly treated except that they were first flooded with their respective non-fluorescent antisera. In some instances sections were stained with non-fluorescent anti-DG prior to application of anti-DF. Alternate frozen sections as well as those prepared after routine formaline fixation were stained with hematoxylin and eosin. In the indirect

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study, sections of normal dog kidney or those prepared from donors remaining kidney were flooded with serum from 8 dogs with transplanted kidneys. Four of these animals exhibited overt transplant rejection and 4 were sacrificed 9 days after transplantation although there was no clinical or biochemical evidence of active rejection. Ultraviolet microscopy was performed with mercury lamp (Scopicon) utilizing Corning exciter filter # 5840 and Wratten 2A barrier filter.

Results. Morphologic alterations in homotransplanted canine kidneys removed prior to, at beginning and terminal phases of rejection were similar to those recounted by others (6,7). Prior to onset of rejection such kidneys appeared histologically normal or exhibited only moderate tubular degeneration and stromal infiltrate of histiocytes, lymphocytes, plasma cells and neutrophils. These changes appeared more severe in animals sacrificed during early periods of rejection and were often accompanied by focal or confluent focal zones of cortical necrosis. In addition, transplanted kidney of animals sacrificed in extremus with elevated BUN, exhibited marked neutrophil infiltration, broad zones of parenchymal lysis and occasional fibrinoid masses within interlobular renal arteries and their smaller branches and less frequently within glomerular tufts (Fig. 1). Sections of major renal arteries were histologically normal. Examination of sections of homotransplanted kidneys and major renal arteries by fluorescent antibody technic failed to reveal preferential localization of canine globulin although the latter was noted at expected sites in control sections. Specific fluorescence did not appear more intense in the inflammatory infiltrate of rejected kidneys than in similar cells not infrequently noted in interstitium of control kidneys. The fibrinoid material in interlobular and arteriolar branches of renal artery and less frequently in glomeruli of such kidneys disclosed specific fluorescence when stained only with anti-DF (Fig. 2).

No affinity for normal renal structure could be detected in sections examined with sera from animals exhibiting transplant rejection by indirect technic.

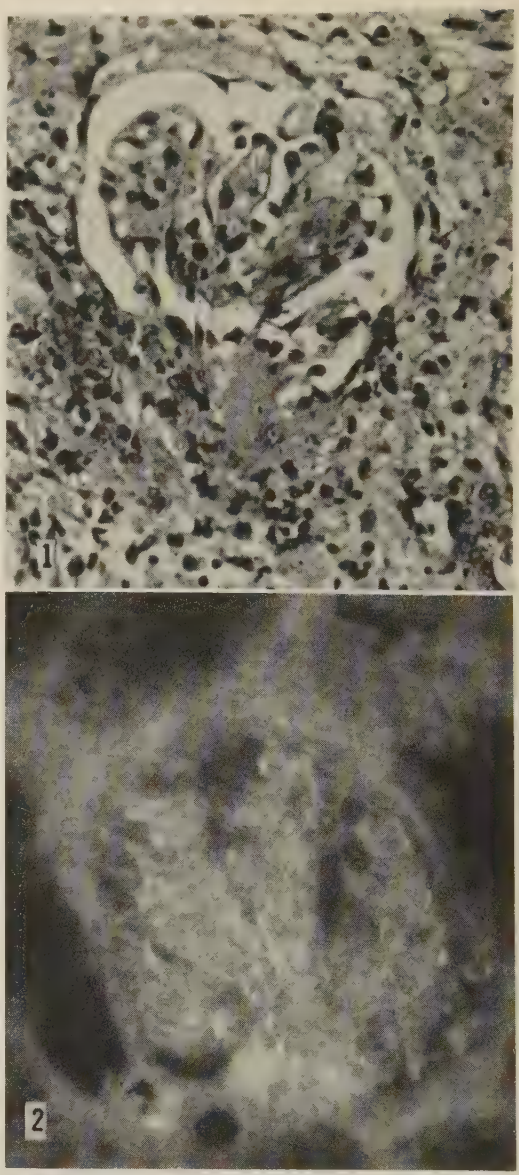

FIG. 1. "Fibrinoid" in glomerular arteriole with extension into capillary tufts. (H. & E. × 280.)

FIG. 2. Fluorescence of material observed in 1 with anti-dog fibrinogen (× 280).

Discussion. Failure to demonstrate abnormal concentrations of globulin in renal homotransplants is consistent with the failure of most workers to detect serum antibodies in this phenomenon, but does not unequivocally indicate their absence. It is conceivable that either antibody in extremely low concentration or antibody with little avidity might not

be detected by this technic. These findings do not exclude the possibility that homotransplant rejection may be of the tuberculin or cellular hypersensitivity type.

Macroscopic and histologic findings of various stages of renal homotransplant rejection are sequentially similar to those observed in renal cortical necrosis associated with certain obstetrical complications and less frequently in men, non-pregnant women and children(8, 9). It resembles the appearance of kidneys in generalized Shwartzman reaction. Demonstration of fibrin composition of vascular and glomerular fibrinoid in some examples of homotransplant rejection is also in keeping with the identification of this material by this immunohistochemical technic in the latter phenomenon by Vazquez and Dixon(10). However, unlike the Shwartzman reaction fibrin thrombi appear to represent a secondary manifestation, being relatively rare and only found in kidneys of those animals considered to be in the terminal phase. Also, we have not observed alterations in factors concerned with blood coagulation in animals experiencing renal homotransplant rejection, but have been considered of pathogenetic significance in the Shwartzman reaction(11). The lack of morphologic or immunohistochemical alterations of major renal arteries does not exclude the possibility that vasospasm of these structures may play a role in pathogenesis of lesions encountered in cortical necrosis as proposed by Sheehan and Moore(9) and demonstrated in renal homotransplantation rejection arteriographically by Dempster(7). Its primacy in this phenomenon remains to be elucidated, however.

Summary. No preferential localization of globulin was noted in rejected homotransplanted canine kidneys studied by fluorescent antibody technic. Indirect variation of this method similarly failed to disclose antibodies exhibiting an affinity for renal structure in serum of recipients exhibiting rejection of renal homotransplants. The fibrin nature of fibrinoid lesions occasionally noted in terminal phases of the rejection phenomenon have been demonstrated. Relationship of pathologic and immunohistochemical alterations of renal homotransplantation rejection and the generalized Shwartzman phenomenon is discussed.

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Corticosteroid Production *in vitro* by the Interrenal Tissue of Killifish, *Fundulus heteroclitus* (Linn).* (24904)

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Earlier work(1) has shown that an adrenocorticosteroid, cortisol, is present in blood obtained from tails of killifish (*Fundulus heteroclitus*). In teleostean fishes the adrenocortical (interrenal) tissue is usually situated in the walls of cardinal veins as they pass through the head kidney. *Fundulus heteroclitus* lacks a left posterior cardinal vein, and the major part of interrenal tissue is found in relation to the right head kidney(2). Evidence that these cells are teleostean adrenocortical tissue is indirect but decisive(3,4), depending on their response to mammalian ACTH and the effects of hypophysectomy. The method of tissue incubation of adrenal tissue has proved a useful tool in the study of corticoid biosynthesis in mammalian preparations(5) and the present investigation resulted from an attempt to apply similar methods to fishes.

Methods. In preliminary study (series A), 45 healthy fish of both sexes were used. Right head kidneys were removed and incubated in 3 batches in manner identical with series B. In series B 163 fish of both sexes were used. Fish for both series were caught at low tide near the mouth of West River, New Haven, June, 1958 (series A) and Sept. 1958 (series B). In series B, right and left head kidneys were removed and pooled separately. Nine batches of each pool, comprising 15-20 head kidneys were incubated for 3 hours at 37°C in 10 ml Krebs Ringer bicarbonate buffer with 200 mg % glucose (pH 7.4) in an atmosphere of 95% O_2 and 5% CO_2 in Dubnoff metabolic incubator. Prior to incubation, 273,493 cpm of progesterone-16-H^3 (representing 0.56 μg) in 0.1 ml propylene glycol were added to each flask. Incubation media of right and left head kidneys were combined separately and each extracted twice with 1.5 volumes of redistilled methylene chloride. The methylene chloride extract was washed once with 1/10th vol. 0.05 N NaOH, twice with redistilled water, evaporated *in vacuo* at 37°C and chromatographed (Fig. 1). Acetylation of the aldosterone area was carried out at room temperature with 25 lambda of 15% acetic anhydride in anhydrous benzene and 20 lambda of absolute pyridine. The reaction was terminated after 48 hrs by adding 0.5 ml absolute ethanol (personal communication). A methylene chloride extract of the mixture was washed with distilled water, dried in air, and applied to paper chromatogram. The acetylated aldosterone area was chromatographed in the cyclohexane-benzene-methanol-water (100:50:100:25) system with adrenosterone as standard reference substance; eluted and rechromatographed in the system cyclohexane-dioxane - methanol - water (100:100:50:250) with Δ^4-androstene-11β-ol-3, 17-dione as a standard. Adrenosterone and Δ^4-androstene-11β-ol-3, 17-dione have the same mobility as aldosterone diacetate in these systems (confirmed in this laboratory using aldosterone - diacetate - C^{14}). Acetylation of

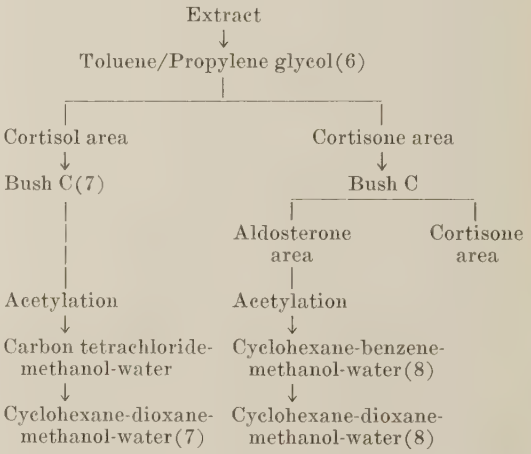

FIG. 1. Chromatographic flow sheet.

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TABLE I. Measurements of Radioactivity at Indicated Points during Chromatographic Analysis. "Control" refers to left head kidneys.

Exp.	Cortisol areas		Cortisone areas		Aldosterone areas	
	Right head kidney cpm	"Control" cpm	Right head kidney cpm	"Control" cpm	Right head kidney cpm	"Control" cpm
Series A, 45 fish						
Counts added			3 × 273,493 cpm			
Counts after Bush C	1456		1359		157	
Series B, 163 fish						
Counts added			9 × 273,493			
Counts after Bush C	11,884	5248	4779	2458	642	1272
No. counts aliquoted	8913	3936				
Counts after E2B	3004	359				
% recovery	34	9				
No. counts acetylated	2250	270			483	954
Counts after acetylation and 2 systems (Fig. 1)	1518	88			462	630
% recovery	67	33			96	66

the cortisol area was carried out in similar manner except that 100% acetic anhydride was used and 20 μg cortisol was added as carrier; then chromatographed in the system carbon tetrachloride - methanol - water (100:100:25); eluted and rechromatographed in the system cyclohexane-dioxane-methanol-water (100:100:50:250). Authentic cortisol monoacetate was run in parallel in both systems.

Results suggest that cortisol, cortisone, and aldosterone were produced (Table I). Series A gave some indication that these 3 corticoids were synthesized from the added precursor. Series B confirmed these findings and added extra evidence for authenticity of the steroids produced. It should be pointed out that a different number of fish were used in the 2 series and since the volume of interrenal tissue is unknown, the data are not directly comparable. Further, the results must take into account 3 facets of experimental technic. Optimal temperature for this species is 15-20°C and therefore incubation of tissues at elevated temperatures may introduce a complicating factor. Also, objections can be raised against the use of a mammalian incubation medium for fish tissue. Finally, it was assumed that progesterone is an intermediary product of adrenocorticosteroidogenesis in fish as it is in mammals. Nevertheless, under these conditions a small percentage of added radioactivity was recovered in steroid areas studied. The head kidneys consist chiefly of lymphoid tissue with only a small amount of adrenocortical cells. It is conceivable, therefore, that only a small portion of the added progesterone-16-H^3 was accessible to the interrenal cells, the excess being metabolized by lymphoid tissue or remaining unaltered.

In incubates of "control" tissue (left head kidneys) there was a striking decrease in amount of cortisol-like steroid after successive chromatographic systems. This emphasizes the necessity for rigorous purification before the authenticity of a steroid can be established. The number of counts/minute and percentage recovery for cortisol from right head kidneys is consistently higher than that of "control" tissue, which suggests that a large portion of radioactivity in "control" incubates can be accounted for by non-specific contamination. The analysis of cortisone was not carried beyond the second chromatography. It is interesting that again the greatest amount of radioactivity was found in the right head kidney incubates. Cortisone has not been found in peripheral blood of Fundulus (1), but it has been identified in a large sample of salmon plasma (unpublished), in adrenal effluents of mammals, the snake and the capon (9,10,11), but not in peripheral blood of humans.

In contrast to findings with cortisol and

cortisone, the amount of radioactivity in aldosterone areas from "control" media was greater than that of right head kidney media. This radioactivity persisted despite 2 chromatographies as the free alcohol, and 2 as the diacetate. However, the magnitude of the differences between right head kidney and "control" results for aldosterone diminished after acetylation. These findings would indicate that either further purification is necessary to establish the identity of the aldosterone believed present or that the "control" tissue can synthesize this mineralocorticoid despite little or no ability to synthesize glucocorticoids. If radioactivity is indicative of aldosterone it raises the question as to whether aldosterone plays a role in electrolyte homeostasis in the fish.

Conclusions. With the above reservations in mind, the following tentative conclusions can be drawn. 1. It seems probable that cortisol, in plasma obtained from *Fundulus heteroclitus,* is synthesized by interrenal tissue of head kidney. 2. Production of cortisol and probably cortisone by both left and right head kidneys suggests that interrenal tissue is present in both, although in lesser amounts on the left. If this is so, no explanation can be given at present for slightly larger amounts of aldosterone produced by left head kidneys. 3. Interrenal tissue of Fundulus possesses enzyme systems, capable of converting progesterone-16-H^3 to tritiated cortisol, cortisone, and aldosterone through biosynthetic pathways not known.

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Bovine Ocular Squamous Cell Carcinoma. III. Tissue Culture Studies of Carcinoma.* (24905)

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Genetic studies of bovine ocular squamous cell carcinoma have demonstrated relationship of incidence of this disease to age of animal and degree of ocular and circumocular pigmentation(1). Studies on pathological anatomy revealed presence of eosinophilic, cytoplasmic inclusion-like bodies in cells of both benign precursor and malignant lesions(2). Tissue culture studies of cells from benign precursor lesions, plaque and papilloma, have shown cytoplasmic changes and inclusions suggestive of presence of a virus(3,4,5,6). Dis-

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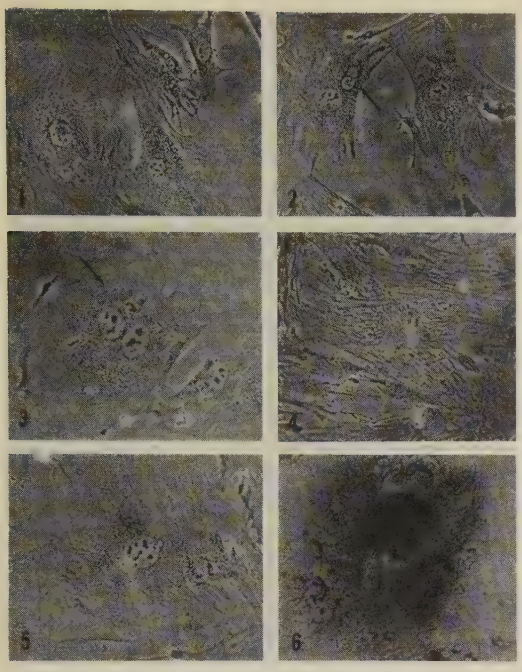
‡ Publication No. 9 of Cancer Eye Study Section.



Photographs were made with phase optics, Zeiss Microscope, Model No. GFL-65, using 35 mm camera attachment.

FIG. 1. Cells derived from bovine ocular squamous cell carcinoma. 3rd passage, 10 day S-M chamber culture. × 130.

FIG. 2. Cells showing cytoplasmic vacuoles with darkly refractile inclusion material. 3rd passage, 10 day S-M chamber culture. × 130.

FIG. 3. Cell showing "crystalline" cytoplasmic inclusion. 8th passage, 6 day S-M chamber culture. × 130.

FIG. 4. Large cell showing well developed tonofibrils. 4th passage, 7 day S-M chamber culture. × 130.

FIG. 5. Large cell showing fine granulation of cytoplasm in perinuclear region. 3rd passage, 10 day S-M chamber culture. × 130.

FIG. 6. May-Gruenwald-Giemsa stained very large cell from 7th passage, 3 day S-M chamber culture of bovine ocular squamous cell carcinoma, showing bizarre pattern of nucleoli. × 325.

appearance, reappearance and bi-laterality of benign precursor lesions may also be suggestive of a viral agent(7). These findings led to tissue culture study of the final, malignant, stage of the disease to determine whether cellular changes similar to those reported in benign precursor lesions are present in cells of bovine squamous cell carcinoma.

Materials and methods. Methods for removal of lesions in the field, their treatment for transportation to and subsequent treatment in the laboratory; also detailed study of cellular outgrowths from benign precursor lesions have already been described(3,4,5,6).

Results. Phase contrast microscopy of cells from carcinoma grown in specially designed chambers(8) has extended and confirmed earlier observations on stained tissue culture preparations in T-30 culture flasks made at low magnifications by bright field microscopy (3,4). Cells derived from carcinomata are epithelioid in character and of 2 sizes (Fig. 1). Large cells measure as much as 0.04 mm in diameter and small cells are of 0.01 mm average diameter. Multinucleated cells occur much less frequently than in cultures derived from benign precursor lesions. Vacuolization of cytoplasm, when encountered (Fig. 2), commonly precedes occurrence of inclusions in the cytoplasm and death of culture. Cytoplasmic inclusions, occasionally observed, are of two types, one preceded by vacuoles, the other irregular in outline and not associated with vacuolization (Fig. 3). The inclusions resemble those found in cultures of cells from papillomata. Cytoplasm of cells derived from squamous cell carcinomata commonly shows well developed tono-fibrils (Fig. 4) and much finer perinuclear granulation (Fig. 5) than that found in cells derived from plaque and papilloma. Occasionally an aggregation of granules occurs in the cytoplasm and is concentrated in the perinuclear region. Cytoplasmic bridging is well marked. Multiple nuclei even in large cells are uncommon. Nucleoli are usually multiple and may exhibit bizarre patterns (Fig. 6). Margination of chromatin, if it occurs, is not definite as in plaque lesion cultures. Cultures derived from carcinomata are stable, number of large and small cells showing little variation over long periods of time and many subcultures. A total of 52 carcinomata have been cultured. Thirty have survived 4 months or less with a maximum of 11 passages. Cytopathogenic changes have occurred in 3 of these cultures between passages one and nine. Twelve cultures have survived for 5 to 8 months with a maximum of 28 passages; 10 of these cultures have shown cytopathogenic changes between passage 4 and 14. Five cultures have been maintained for more than 12 months and 43 passages. Cytopathogenic

changes have been noted in 4 of these cultures after the 15th but not after the 27th passage. Cytopathogenic changes observed in cultures of carcinomata do not lead to complete loss of culture. It is possible to preserve some cells and gradually regenerate the cell line. When changes occur early, between passages 1 and 7, it is not possible to save the culture. Seventeen cultures derived from bovine ocular squamous cell carcinoma are presently maintained. Average number of passages during more than 6 months is 25. In two cases 30 passages and in one case 43 passages have been possible in cell lines grown continuously for 10 months.

Summary. Behavior and properties of cells derived from bovine ocular squamous cell carcinoma grown *in vitro* are described. In these cells, unlike those from benign precursor lesions, plaque and pappilloma, cytoplasmic inclusions have seldom been observed. Multiple nuclei and margination of chromatin have been found only in rare instances. Changes, normally associated with cells of cultures of plaque and papilloma, have been found in cells from lesions diagnosed as transitional from plaque or papilloma to carcinoma.

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Carcinogenic Effects of Petroleum Asphalt.* (24906)

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Evidence indicates that some petroleum products are carcinogenic and that the carcinogenic effects of these products may vary with geographical source and refining procedures to which the crude oils have been subjected. Because of the enormous increase in use of petroleum asphalts, the question occurs as to whether these materials may be of importance in cancer causation. It is reported (1) that asphalt tonnage in the United States increased from 20,000 tons in 1902 to 9,000,000 tons in 1946. Interpretation of previous work in this field is often confused by using the terms pitch, tar and asphalt without defining precisely what each term means. An examination of the literature shows no reference to studies of carcinogenic effects of commercially manufactured petroleum asphalt, as defined here, used for paving, roofing, waterproofing, etc. This investigation seeks to answer the question: Is petroleum asphalt, as defined in the following paragraph and made from western United States crude oils, carcinogenic under the conditions of these experiments?

Methods. Asphalts, as used here, are (2): "Black to dark brown solid or semi-solid cementitious materials which gradually liquefy when heated, in which the predominating constituents are bitumens all of which occur in the solid or semi-solid form in nature or are obtained by refining petroleum, or are combinations of the bitumens mentioned with each other or with petroleum or derivatives thereof." "Bitumen is that portion of petroleum asphalt and tar products which will dissolve completely in carbon disulphide(3)." Manufactured, as contrasted with natural, asphalt is produced by distilling off the lower boiling point fractions of crude petroleum—gasoline,

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kerosene, and a variety of other oils—the residue is asphalt. In general, 2 methods of distillation are used. In steam-blown (straight-run) asphalt, distillation is accomplished in tubular stills. Distillation is aided by admitting steam and the lighter fractions are removed from the top of the column and the asphaltic fractions are continuously drawn off at the bottom. In this method of distillation, exposure to high temperatures which might result in cracking and alteration of the chemistry of the crude oil is avoided. The second method of distillation results in cracking and chemical alterations in the crude oil because of the high temperatures employed. Shell stills are used and hot air is blown into the material through perforated pipes. Such asphalts are known as "air-blown" or "oxidized." C-57 black mice, purchased from Rockland Farms, New City, N. Y., were the test animals used. The asphalt used is a pooled lot from 6 different samples supplied by Southern California refiners. Both steam and air-blown asphalts are represented in the pooled material. The mice have been exposed to asphalt in 2 ways: Group I, consisting originally of 68 mice (32 males and 36 females) have had asphalt, rendered liquid enough for handling by addition of benzene, applied with a glass rod to the skin of the interscapular region 2 times weekly. Group II, consisting of 63 mice (31 males and 32 females) were similarly treated with benzene alone. Group III, made up of 33 males and 29 females were injected subcutaneously in the interscapular region 2 times weekly with .2 cc of a 1% suspension of asphalt in olive oil. Frequency of injection was reduced to once weekly after 41 weeks because the volume of material under the skin became excessive. Group IV composed of 32 males and 28 females were treated as Group III except that olive oil alone was injected.

Results. Group I: There have been 12 epidermoid carcinomas form at site of painting. Fig. 1 A and B show the characteristics of the cancers in this group. Four(4) mice of this group are alive with well-developed papillomas and one mouse died with a prominent papilloma. Formation of the cancer was preceded by epilation, dryness, scaling and papilloma formation. Fifty-four weeks elapsed before appearance of the first epidermoid carcinoma. Slight epilation, dryness and scaling have been the only changes observed in Group II.

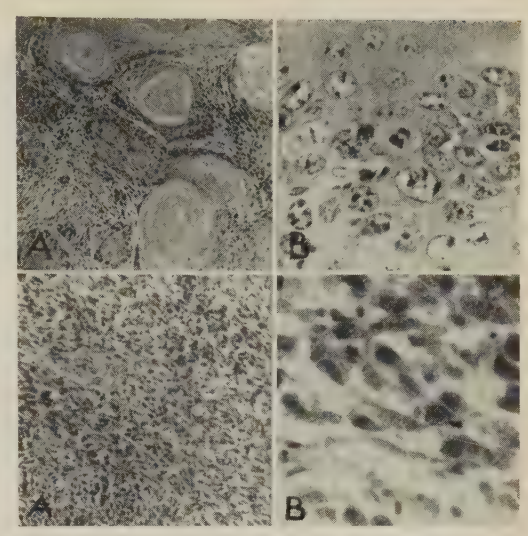

FIG. 1 (top). Epidermoid carcinoma, H and E stain. A, × 80; B, × 360.
FIG. 2 (bottom). Fibrosarcoma, H and E stain. A, × 160; B, × 720.

Group III: Eight sarcomas have occurred at site of injection. One of these is a rhabdomyosarcoma and the remainder are fibrosarcomas. Fig. 2, A and B show a representative example of these tumors. Thus far, distant metastasis has not been seen. One sarcoma has been successfully transplanted to a female C-57 black mouse. Thirty-six weeks of injection were required before occurrence of the first sarcoma. Thus far, Group IV has shown no evidence of tumor formation.

Discussion. These carcinomas and sarcomas conform to all of the conventional histological criteria for malignancy. One of the sarcomas has been successfully transplanted into a female mouse of the same strain. Transplantation has been attempted with but 2 of the sarcomas and one of the carcinomas. All tumors have occurred at site of treatment and no similar tumors have occurred in any of the control groups. One benzene-painted control died and microscopic examination of the organs disclosed leukemic infiltration of the salivary glands and the lungs.

Conclusions. A pooled sample of petroleum asphalt manufactured from western U. S.

crude oils contains a substance or substances which will cause epidermoid carcinomas when applied externally to the skin of C-57 black mice and sarcomas when injected subcutaneously into the same strain.

We are grateful to Dr. Raymond L. Teplitz who rendered important service by microscopic examination of specimens.

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Studies in Wound Healing. III. Contraction in Vit. C Deficiency. (24907)

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The closure of full thickness skin defects in mammals by spontaneous approximation of the wound edges—wound contraction—has been demonstrated to result from forces localized in the wound margins(4,8). Although the site of origin of the contraction process has been clearly identified, the mechanism remains obscure. Histologic study of the wound margin in the guinea pig has revealed an inactive dermis but a highly cellular area of proliferation of loose subdermal connective tissue (unpublished data), suggesting that the latter is the active layer within the margin. While our studies indicated that the collagen fibers composing the central mass of wound granulations were unrelated to the contraction process(4,8), they did not clarify the roles of individual components of the new forming connective tissue in the marginal area, nor did they exclude a possible function for new formed collagen fibers in this region. Experimental scurvy offers a method for partial dissection of components of connective tissue by inhibition of collagen formation(5,7,10). Abercrombie, Flint and James(1) stated that the contraction occurring in skin defects in scorbutic guinea pigs in the first 10 days after wounding did not differ significantly from that in normal animals. Woessner(9), on the other hand, concluded that "contraction took place normally in wounds of animals on ascorbic acid supplemented diets, but in scorbutic animals very little contraction took place." Edwards and Dunphy commented on conflicting reports of contraction in scurvy(3). This point is of obvious importance in determining relative roles of cells and fibers in the "picture frame" mechanism; we report here the results of systematic study of contraction in skin wounds in scorbutic guinea pigs.

Methods. Male guinea pigs weighing 300-400 g were used. The control animals were placed either on a standard laboratory diet of Purina pellets or on a MacDonald #5 Scorbutigenic Diet(2),‡ receiving supplementary Vit. A, B complex, D, and, in addition, ascorbic acid. Experimental animals were placed on the same diet omitting ascorbic acid for 7 and 12 days prior to wounding. Wounds 2 cm square were made on each side of the back through skin and panniculus carnosus. Wound outlines were traced initially and at intervals of one to 3 days. Areas were determined by planimetry. *Wounds without dressings.* For the control series of guinea pigs with undressed wounds and intact scabs, our previously published data(4) were used. In a second control series scabs were removed at

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‡ Dr. W. VanB. Robertson, Coll. of Med., Univ. of Vermont, made available generous supply of this diet.

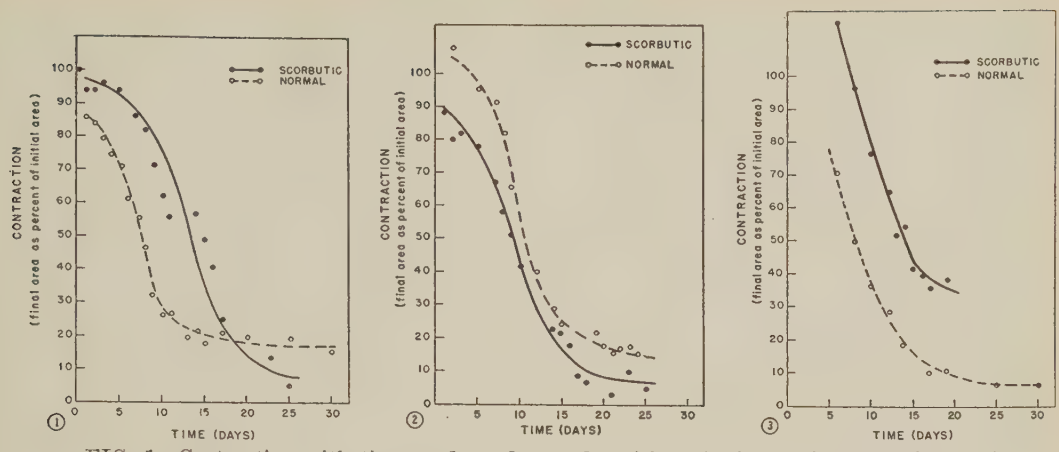

FIG. 1. Contraction with time, undressed wounds, with scabs intact, in scorbutic and in normal guinea pigs as indicated. Points represent averaged measurements.

FIG. 2. Contraction with time, undressed wounds, where scabs have been repeatedly removed, in scorbutic and in normal guinea pigs. There is no significant difference between these 2 curves. Hence apparent reversal of scorbutic and normal curves when compared with Fig. 1, where scorbutic contraction was delayed, is not significant.

FIG. 3. Contraction with time, dressed wounds, scorbutic and normal animals.

frequent intervals from 8 wounds, care being taken not to disrupt the wound margins. In scorbutic test animals 7 wounds with scabs intact, and 11 wounds with scabs removed at intervals of 2 or 3 days were followed. *Wounds with dressings.* Sterile vaseline gauze dressings were held in place by light plaster jackets. Four normal animals, 4 animals on a scorbutigenic diet for 7 days prior to wounding and 5 on the diet for 12 days prior to wounding were studied. Body weight was followed as an index of general condition. No infection occurred in these animals.

Results. Criteria of scurvy. A state of total scurvy was established by histologic examination of the incisor tooth roots as described by Crampton(2) at times ranging from 7 days on the Vit. C deficient diet. Further criteria of the scorbutic state were obtained by demonstrating histologic absence (VanGieson's stain) of collagen in the scorbutic wounds at times when it was clearly demonstrable in the normal. Chemical analysis of the scorbutic granulation tissue for hydroxyproline content(6) revealed 1.4 γ per mg of dry tissue at 9 days, and 1.0 γ per mg at 12 days as compared with 25 γ per mg at 9 days and 35 γ per mg at 12 days in the normal.

Wounds without dressings. Characteristic thick scabs formed in scorbutic wounds and remained in place several days longer than normally if undisturbed. The curve of contraction when the scab was left intact (Fig. 1) showed a delay in contraction in the first 5 days in contrast with the prompt decrease in wound area occurring in the normal. However, once contraction began, its rate was identical with that of the control. We have previously noted that repeated removal of scabs from normal wounds did not significantly alter the contraction pattern(8). This is again demonstrated by comparing the curves of normal wound contraction (Figs. 1 and 2) where the scabs have been left intact and where they have been removed. When scabs were repeatedly removed from scorbutic wounds, the contraction curve varied little from the normal (Fig. 2).

Wounds with dressings. Further confirmation of the ability of the scorbutic wound to contract actively was obtained by measurements on wounds kept moist by dressings which prevented drying and crusting which causes scab formation. Again, (Fig. 3) the slope of the contraction curve in scurvy is much the same as the normal. At 6 days (the earliest feasible period for observing this type of wound) the area was enlarged as compared with the normal; again contraction was

delayed 5 or 6 days. The behavior of wounds in animals on the scorbutigenic diet for 7 and 12 days prior to wounding was very similar. It is important to record the observation that the contracting scorbutic wound margin at any time could be readily detached from the base, causing immediate distraction. The wound margin was much more firmly bound in the normal.

Discussion. It must be emphasized that the term "wound contraction" is used here to describe that early phase of closure of tissue defects characterized by the movement of preformed tissues, and does not describe the late changes in scar tissue shortening. This latter process is probably different in origin and dependent on collagen fibers of the new formed connective tissue (although direct evidence is presently lacking).

The fact that contraction, except for an initial delay, proceeds in an almost normal manner in scorbutus is consistent with the hypothesis that the actual movement inward of the wound edge is dependent upon directed cell migration as originally suggested by Abercrombie, Flint and James(1), and by Grillo, Watts and Gross(4,8). The markedly different strength of attachment of the wound margins in normal and scorbutic wounds suggests that the role of collagen in this process, if any, is to produce a temporary and continually remodelled tethering of the wound margin to the base. Thus, as the margin progresses inward, collagen fibers might be laid down in advance and removed or detached as the wound edges pass overhead, a phenomenon suggested by our previous observation(4) that normally firm attachment was found only at the wound margin and relatively little collagen could be demonstrated peripheral to the margin beneath the advancing skin.

Summary. Full thickness skin wounds in scorbutic guinea pigs contract at same rate during the first 2 weeks of healing as do those in normal animals. There appears to be a delay in initiation of contraction. Significance of these observations is discussed.

The authors thank Martha Morton for valuable technical assistance.

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Interference Between Certain Neurotropic Viruses in Tissue Culture.* (24908)

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Interference between Col SK virus and poliomyelitis virus, which occurs in monkeys, guinea pigs or in hamsters upon infection with both viruses, has previously been described (1-6). More recently, tissue culture methods have been employed in quantitative studies of interference reactions between related or unrelated viruses(7-13). Since several strains of the Col SK virus group can be propagated on certain cell lines with definite cytopathogenic effects(14), it became of interest to study interference phenomena between these agents and other neurotropic viruses on suit-

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able tissue culture substrates. As has been reported before(14c), tissue cultures of L cells, HeLa cells and KB cells support cytopathogenic growth of EMC virus but not of Col SK virus which cannot be maintained over serial passages on the corresponding tissue culture media; this discrepancy in growth requirements of the 2 viruses extends also to human amnion cells. Further experiments showed that, notwithstanding the absence of cytopathogenic growth, considerable amounts of Col SK virus are adsorbed on the cellular surface of HeLa cultures, provided the test dose of virus is kept within reasonable limits. This was determined by titration in mice of viral supernatant fluids, removed from cell sheets after variable periods of contact at 37°C, in comparison with the titration of viral fluids obtained from cell-free controls. The actual amount of a test dose of 10^5 LD_{50} of Col SK virus, which was adsorbed on about 5 million HeLa cells after an interval of 4 hours, was calculated to be of the order of 2 logs. Therefore, an opportunity presented itself to utilize non-cytopathogenic Col SK virus as interfering agent against subsequent challenge with other fully cytopathogenic neurotropic viruses on various cell lines.

Materials and methods. The following viruses† were used: Col SK virus, EMC virus, Poliomyelitis virus (Type I: Mahoney, Brunhilde; Type II: Y-SK, MEF_1; Type III: Saukett) and Coxsackie virus (Group B, Type 5). Col SK virus was employed as brain passage virus harvested from paralyzed mice. EMC virus was harvested from infected L cell cultures. The poliomyelitis and Coxsackie viruses were tissue culture fluids harvested from infected HeLa cell cultures. L cell cultures were used for interference experiments between Col SK and EMC virus; the medium for L cells was Hanks' solution with 40% horse serum and 20% chick embryo extract (plus antibiotics). Interference reactions between Col SK virus and poliomyelitis or Coxsackie viruses were studied on HeLa cells or on human amnion cells. The HeLa cells were available in 2 sublines I and II which differed in their sensitivity to cytopathogenic growth of EMC, poliomyelitis, or Coxsackie viruses, the titers of all 3 viruses being consistently at least one log higher with the more sensitive cell strain. HeLa and amnion cells were propagated in a medium consisting of 20% horse serum and 10% human serum in Hanks' balanced salt solution which contained 0.1% yeast extract (plus antibiotics). After 4-7 days growth the cells were trypsinized and suspended (100,000-200,000 cells per ml) in Scherer's maintenance solution containing 10-20% horse serum. All cell suspensions were then distributed, in 0.5 ml volume, into rubber-stoppered stationary tubes and 2-day-old sheets, after replacement of the medium, were used for interference tests. The basic experimental procedure adopted was as follows: For each experiment 7 groups of tissue culture tubes were set up, with 5 tubes in a group. A constant amount of Col SK virus, *i.e.* 0.1 ml of a 1:125 dilution, was added to each tube containing 0.4 ml of maintenance fluid; higher concentrations of Col SK virus were toxic for cells because of their content of mouse brain tissue. After contact of 4 hours at 37°C, the fluids were drained and replaced by 0.4 ml of fresh maintenance fluid; parallel tests showed that the results were essentially the same with cell sheets which had been washed 3 times. Following adsorption the cells were infected with 0.1 ml of the challenge virus in 7 serial dilutions progressing in 5-fold steps to the endpoint of cytopathogenicity. All tubes were then reincubated. The challenge virus was titrated simultaneously over the same range of dilutions under similar experimental conditions, except that a 1:125 suspension of normal mouse brain was added as first inoculum. Thus, 35 tubes were used for the test and an equal number for the control, making a total of 70 tubes for each interference experiment. All cell sheets were examined microscopically on the third and fourth day after infection, the endpoints of cytopathogenicity were determined by the Reed-Muench method, and the titers were ex-

† Poliomyelitis viruses and Coxsackie virus were obtained through courtesy of Dr. H. Winsser, Albany, N. Y. We are indebted to Dr. J. Syverton, Minneapolis, and to Dr. B. Mandel, N. Y. City, for supplying the 2 lines of HeLa cell cultures; L cells and the stable strain of human amnion cells were kindly provided by Dr. M. Holden of this Department.

TABLE I. Interference between Col SK Virus and EMC Virus on L Cells.

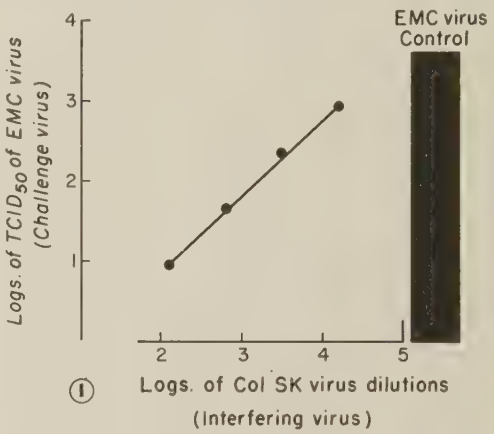
Interfering virus dilutions	Challenge virus 1:125-1:390625	L cells: EMC control	L cells: Interference test	L cells: Rate of interference
		TCID$_{50}$ (logs)		
Col SK 1:125	EMC	3.60	.97	2.63
1:625	"	"	1.67	1.93
1:3125	"	"	2.34	1.26
1:15625	"	"	2.90	.70

pressed as TC ID$_{50}$ per 0.1 ml of culture fluid. It was therefore possible to calculate rate of interference as logs of challenge virus suppressed in each of the various interference systems. With the Col SK-EMC and Col SK-Mahoney interference systems the basic procedure was extended to include further quantitative tests in which Col SK virus was used as interfering agent in serial 5-fold dilutions over a range from 1:125 to 1:15,625. The results of these experiments are brought together in Tables I and II.

Results. The data given in Table I indicate that effective interference between Col SK and EMC virus occurred on L cells. The degree of this interference ranged from suppression of 0.7 log to 2.63 logs of challenge virus, depending on dose of Col SK virus used during the initial phase of the experiment. When the figures are plotted log for log, as in Fig. 1, it will be seen that interference between the 2 viruses proceeded as a first order reaction over the range of dilutions tested. None of the poliomyelitis viruses, when placed in contact with L cells, interfered with subsequent production of EMC virus.

It appears from Table II that effective interference occurred also between Col SK virus and poliomyelitis or Coxsackie viruses with HeLa cells, subline I. The degree of interference varied from suppression of about 1 log to almost 3 logs of challenge virus. In the case of the Col SK-Mahoney virus system, a similar quantitative relationship between amount of interfering virus and amount of suppressed challenge virus could be demonstrated as for the Col SK-EMC system (Fig. 2). Interference between Col SK and poliomyelitis virus was less marked on amnion cells since only about 1½ logs of challenge virus were suppressed. Minimal interference, or no interference at all, was observed with the more sensitive subline II of HeLa cells.

After the basic phenomenon was established, further experiments were concerned with a more detailed study of the mechanism of the interference reaction. The Col SK-Mahoney virus system on HeLa cells was



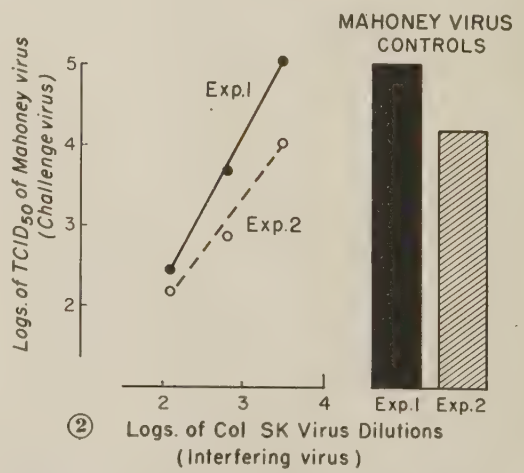


FIG. 1. Interference between Col SK and EMC virus in L cell tissue culture.

FIG. 2. Interference between Col SK and poliomyelitis virus in HeLa cell tissue culture.

TABLE II. Interference between Col SK Virus and Poliomyelitis or Coxsackie Virus on Various Tissue Culture Substrates.

Interfering virus	Challenge virus	HeLa cells (subline I)			HeLa cells (subline II)			Amnion cells		
		Control	Interference test	Rate of interference	Control	Interference test	Rate of interference	Control	Interference test	Rate of interference
		$TCID_{50}$ (logs)								
Col SK 1:125	Mahoney	4.47	2.44	2.03	5.60	5.25	.35	5.24	3.84	1.40
"	"	4.37	1.57	2.80						
"	"	4.53	1.63	2.90						
"	Brunhilde	4.53	3.26	1.27	5.24	5.24	0			
"	MEF_1	2.80	1.91	.89	3.99	3.34	.65	1.86	.34	1.52
"	Y-SK	4.63	1.74	2.89						
"	Sauckett	4.89	3.03	1.86	5.60	5.60	0	3.03	1.63	1.40
"	Coxsackie B_5	4.61	2.10	2.51				4.19	3.03	1.16

chosen as a model for this investigation, using Col SK virus in a constant dose of 1:125 for interference against challenge with Mahoney virus over a range from 1 to 100 TC ID_{50}. Several interesting observations were made. Thus, it was found that Col SK virus (1:125) which had been heated for 10 min at 53°C —a procedure that reduced the infectivity titer in mice from 10^{-9} to less than 10^{-3}— gave no evidence of interference. This result agrees with the observation that a dose of between 7 and 8 logs of active Col SK virus is required for suppression of about 100 TC ID_{50} of Mahoney virus. In other tests in which length of time allowed for contact between Col SK virus and cells varied over a range from 15, 30, 60, 120 minutes; 4 hours; 24 hours and 3 days, no interference at all occurred before 4 hours; the reaction was maximal at this time and equal protection was obtained at later intervals, up to 3 days. This finding is in harmony with rate of adsorption of Col SK virus on HeLa cells. The problem as to site of interference was studied by examining the effect of receptor-destroying enzyme on the reaction. It was found that previous treatment of HeLa cells with *Vibrio cholerae* RDE (1:50 dilution for 1 hour at 37°C) completely abolished the interfering action of Col SK virus. The outcome of this experiment is in line with what might have been expected, namely that interference does not take place unless Col SK virus has been adsorbed on the cells(15). However, it leaves open the question whether interference occurs on the cellular surface, or intracellularly. Finally, a number of tests were run with bottle cultures of HeLa cells in an effort to follow the interfering ability of Col SK virus at various intervals after its adsorption. In these experiments HeLa cell sheets were covered with 7 cc of a 1:100 dilution of Col SK virus in maintenance fluid. After contact between virus and cells for 4 hours at 37°C, the supernatant was removed and cells were thoroughly washed, including washing with specific antiserum, to remove any traces of unadsorbed virus. The cells were then covered with fresh maintenance fluid and the bottles were reincubated. The original virus di-

TABLE III. Loss and Re-appearance of Interfering Ability of Col SK Virus following Adsorption of Virus and after Its Subsequent Release from HeLa Cells.

Preparation	Interfering virus—Col SK: Infectivity LD_{50} (logs)	Challenge virus—Mahoney: Control	Challenge virus—Mahoney: Interference test	Challenge virus—Mahoney: Rate of interference
		$TCID_{50}$ (logs)		
Original virus dilution	8	4.77	3.13	1.64
Supernatant after 4 hr contact with cells		"	4.77	0
" " washing cells	1	"	"	0
" " 6 hr re-incubation	5	"	"	0
" " 12 hr "	6	"	3.93	.84
" " 24 hr "	8	"	3.37	1.40

lution before adsorption as well as supernatants withdrawn at several points after adsorption were titrated by standard methods in HeLa cell tubes for interference against Mahoney virus; fluids collected after washing the cells were also titrated in mice for infectivity. Table III gives the results of one representative experiment.

The data show that the interfering power of the supernatant was totally lost after 4 hours contact between Col SK virus and cells but that it reappeared between 12 and 24 hours following reincubation. Comparative tests in mice did not permit demonstration of any loss of infectivity through adsorption because of the excessive dose of virus used; however, it is clear that reappearance of the interfering principle, after washing of the cells, was paralleled by a corresponding release of infectious virus. Interestingly enough, both interfering power and infectivity of a supernatant, harvested at the 24-hour interval and then placed in contact with a new sheet of HeLa cells, could be shown to go through another phase of adsorption, but not of release.

Discussion. Our observations indicate that non-cytopathogenic Col SK virus interferes with subsequent growth of cytopathogenic EMC virus on L cells in tissue culture. Furthermore, it appears that magnitude of the interfering effect is directly proportional to amount of Col SK virus used for adsorption. Our results compare with what is known of interference phenomena among other related viruses in tissue culture experiments and are quite similar to those reported by Dinter(12) on interference between non-cytopathogenic and cytopathogenic strains of foot and mouth disease virus. Of greater importance, perhaps, is that definite interference can also be demonstrated on HeLa cells between Col SK virus and the 3 serological types of poliomyelitis virus or Coxsackie Group B virus. This fact serves to amplify earlier observations by other workers on occurrence of interference between these viruses in animal or tissue culture tests.

The actual mechanism of this interference, especially its site on the host cell, is not known. However, since Col SK virus uses cell receptors which are different from those used by poliomyelitis virus—one being susceptible to destruction by RDE, the other resistant to the action of the same enzyme(15)—suppression of virus multiplication may not occur on the cellular surface but intracellularly by blocking certain intrinsic metabolites at an early step in the process of viral synthesis. It remains for further experiments to clarify this problem. Recent work by Isaacs(10) suggests that, when heat-inactivated influenza virus is used as interfering agent against active influenza virus on chick chorio-allantoic membranes *in vitro,* a non-infectious, non-specific product of the interference phenomenon ("interferon") is formed which is capable by itself of inducing protection. Our experiments with active Col SK virus yielded no evidence that interference can be ascribed to any factor other than virus itself.

Summary. 1) Interference between non-cytopathogenic Col SK virus and cytopathogenic EMC virus occurred on L cells in tissue culture, causing suppression of 0.7 to 2.63 logs of challenge virus depending on the dose of interfering virus used. 2) Interference also

occurred between Col SK virus and poliomyelitis or Coxsackie Group B virus on HeLa cells, causing suppression of about 1 to 3 logs of challenge virus depending on the dose of interfering virus used. 3) The mechanism of the interference reaction is discussed.

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Bussuquara, A New Arthropod-Borne Virus.* (24909)

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Sentinel monkeys have been used extensively by the Belém Virus Laboratory in attempts to isolate arthropod-borne (arbor) viruses. In this paper are described serologic studies that led to characterization of an agent (An 4073) thus isolated as a new, hitherto undescribed arbor virus, which it is proposed to name Bussuquara. *Circumstances of isolation and early studies.* Bussuquara virus was isolated in the forest of the Inst. Agronomico do Norte, Belém. Blood from a sentinel howler monkey (*Alouatta beelzebul*) was inoculated intracerebrally into 3-day-old mice. Beginning on sixth day, some inoculated mice showed signs of illness. Brain tissue emulsions from these sick animals were in turn pathogenic for new suckling mice on intracerebral inoculation. The agent isolated was serially propagated by intracerebral passage in suckling mice. It readily passed through Seitz filters, and cultures of the infectious brain emulsions proved sterile on bacteriological media. Three days later the virus was again isolated from blood of the monkey. Sixteen days after second sample of infected blood was taken, the monkey died, and as serum from the moribund animal was markedly icteric, the liver was submitted to Dr. Madureira Para for histopathological examination. He reported lesions compatible with a diagnosis of yellow fever. Although the isolated virus was not neutralized by a known yellow fever immune serum, serum samples from adult mice that had survived intracerebral inoculation of this virus reacted in a hemagglutination-inhibition (HI) test with several antigens of group B arbor viruses at dilutions up to 1:160. To study the immunologic relationship of the agent further, the following experiments were made.

Methods. Immunologic characterization. No special effort was made to determine biological properties of the agent beyond what was necessary to characterize it

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serologically.‡ In second to fifth mouse passages, the virus was pathogenic for newborn mice on intracerebral and intraperitoneal inoculation with a titer around 10^{-8}, and for adult mice on intracerebral inoculation with a titer of approximately 10^{-7}. Average survival time of newborn mice was 4 or 5 days and that of young adults around 6 days, when an inoculum of 10^{-1} or 10^{-2} dilution of virus was used. Immunologic characterization of the virus was done by HI, complement-fixation (CF) and neutralization tests. Procedures for the HI test have been described(1). Immune sera were prepared by inoculation of the respective viruses either once or repeatedly from 3 to 6 times. All multiple-injection sera, as well as single-injection sera against Bussuquara and bat salivary gland (BSG) viruses, were obtained from mice after intraperitoneal inoculation; the remaining single-injection sera were obtained from guinea pigs inoculated intracerebrally. Except when otherwise indicated, acetone-ether extracted antigens were used in HI and CF tests. In CF tests, extensive use was made of box- or checker-board-titration system, in which each serum was tested in increasing 2-fold dilutions beginning at 1:4 (occasionally 1:2) and extending to 1:512 against each antigen similarly diluted. Our results of box-titration CF test are expressed as fractions, the numerator being titer of serum and the denominator, titer of antigen. Neutralization tests were carried out by intracerebral inoculation into 3- to 6-day-old mice of mixtures of equal parts of undiluted serum and dilutions of virus. Mixtures were incubated at 37°C for 1 hour before inoculation.

Results. Extracts to be used as antigens in a CF test were prepared from brain and from liver tissue of newborn mice infected with Bussuquara virus by centrifugation at 2000 rpm for 20 minutes of a 10% emulsion of each tissue in physiological salt solution. The supernatant fluids, designated undiluted antigens, were tested in dilutions 1:2, 1:8 and 1:16 against 4 serum pools, each in 2-fold dilutions ranging from 1:2 to 1:64. These 4 pools contained equal parts of hyperimmune mouse serum against individual arbor viruses as follows: pool 1—(group A) Mayaro, Venezuelan equine encephalitis, western equine encephalitis and eastern equine encephalitis; pool 2—(group B) Ilhéus, dengue type 1, yellow fever and Zika; pool 3—(group C) Oriboca, Murutucu and Apeu; and pool 4—(miscellaneous) Bunyamwera, Be An 277,§ Be H 151 and Tr 8900. Of the 8 antigen-serum combinations, only one reacted positively, namely, serum pool group B and brain tissue extract; the serum gave a titer of 1:8 and the antigen reacted at a dilution of 1:2. This result confirmed the earlier indication that Bussuquara is a group B virus.

Additional evidence was secured by further HI tests. Hemagglutinating antigens with Bussuquara were prepared from infected brain tissues of suckling mice. The antigen was active at pH range of 6.4 to 7.6, with optimal zone between 6.8 and 7.3; as a rule the antigen was used at pH 7.0 and incubated at either 22°C or 37°C. Under these conditions and on lucite trays, titer of antigen was of the order of 1:300. Twelve known immune mouse sera against 12 arbor viruses were tested simultaneously with 4 or 8 units of Bussuquara antigen and with their homologous antigens; 7 of the sera represented group A arbor viruses, 4 sera group B agents and one serum Bunyamwera virus. The results of this test were as follows, homologous titer being given first: Group A—Chikungunya, 1:160, 0; eastern equine encephalitis, 1:320, 0; Mayaro, 1:640, 0; Semliki Forest, 1:2560, 0; western equine encephalitis, 1:1280, 0; AMM 2021, 1:640, 0; AMM 2354, 1:320, 0; Group B—BSG, 1:160, 1:80; St. Louis encephalitis (SLE), 1:80, 1:20; West Nile (WN), 1:640, 1:160; yellow fever, 1:640; 1:320; and finally, Bunyamwera, 1:640, 0.

‡ Bussuquara virus has been maintained serially by inoculation of mosquitoes, thus fulfilling one of the essential experimental criteria for arthropod-borne nature of a virus (Dr. Loring Whitman, personal communication).

§ Some viruses mentioned in this paper have not yet been described by the authors responsible for their isolation: Be An 277 and Be H 151, isolated by Dr. Ottis R. Causey; Tr 8900, by Drs. Wilbur G. Downs and Charles R. Anderson; AMM 2021 and AMM 2354, by Dr. E. L. Buescher; and SA H 336, by Dr. Kenneth C. Smithburn.

TABLE I. Complement-Fixation Test.

Serum	Antigen				
	Buss.	SLE	Ilhéus	BSG	SA H 336
Bussuquara	1:64/1:16	1:8/1:32	1:4/1:16	0/0	1:4/1:8
St. Louis encephalitis	1:4/1:4	1:128/1:256			
Ilhéus	0/0		1:256/1:512		
Bat salivary gland	1:4/1:2			1:64/1:64	
SA H 336	0/0				1:32/1:256

These results leave no doubt that Bussuquara virus belongs in group B of the arbor viruses.

With the group affiliation of Bussuquara virus thus established, attempts were made to determine whether it was a new agent, *i.e.*, distinguishable from previously described group B viruses. For this the CF test was used extensively, since previous experience had shown it to be in general more specific than HI in group B(2). Multiple-injection mouse sera were used and, with one exception to be noted later, complete box titrations as described above were done. In the 3 complete box-titration tests carried out several antigen-serum systems were included in addition to Bussuquara, which was present in all; samples of Bussuquara antigen and serum were of different lots in the different tests. In the first test, the homologous titers of Bussuquara serum and antigen were 1:16 and 1:8, respectively; in the other 2 tests, 1:64 and 1:16. In no instance did Bussuquara antigens or sera react with antigens or antisera prepared from yellow fever (French neurotropic strain), dengue type 1, dengue type 2, Japanese encephalitis or WN (Egypt 101 strain) viruses, even though these systems were shown to have titers of 1:32 to 1:128 for the sera and 1:64 to 1:256 for the antigens.

Table I gives results obtained when Bussuquara antigens or sera were tested against similar reagents prepared from SLE, BSG, Ilhéus and SA H 336 viruses. It can be seen that sera from mice immune to these 4 viruses either failed to react with Bussuquara antigen, or at best reacted to 1/16 or less of their homologous titer, even then only in the presence of an excess of antigen. Similarly, Bussuquara virus immune serum was at best but weakly reactive with the other antigens and again only when these were in excess quantity.

Cross reactions of the type shown in Table I could not be detected in all cases when the systems were tested owing probably to the low titers of the cross reaction. Thus, in 4 tests with 2 different SLE immune sera, only the positive cross reaction shown in Table I occurred, and in 3 tests with 2 different BSG immune sera there was also only one positive cross reaction. On the other hand, all 4 different samples of Bussuquara virus immune serum reacted with SLE antigen, but only one sample of 2 tested reacted with Ilhéus and SA H 336 antigens.

An additional CF test was carried out to compare Bussuquara virus with other group B agents not included in previous tests. In this test, Bussuquara antigen was used in dilutions 1:2.5, 1:5, 1:10 and 1:20; all other antigens in dilutions 1:8 and 1:16. Immune sera were used in serial 2-fold dilutions beginning at 1:2 for Bussuquara and at 1:4 for all the other viruses. The Bussuquara immune serum titrated 1:16 and the antigen 1:10. Homologous titers for the other immune sera were: Uganda S, 1:32; Zika, Russian spring-summer encephalitis and Spondweni, 1:64; and Japanese encephalitis, Ntaya and Wesselsbron, 1:128. Only one of these sera, Spondweni, reacted with the Bussuquara antigen and then only in a titer of 1:4. The Bussuquara serum failed to react with any of the antigens included in the test. While it is possible that some cross reactions might have been demonstrable had sera or antigens been more potent, the results of the CF tests show conclusively that Bussuquara virus is antigenically distinct from any of the other group B viruses included in the tests.

Studies by HI did not separate Bussuquara virus as sharply from other group B viruses as did CF tests, particularly when the immune sera used were from mice repeatedly inoculated. Since it has been reported(2) that sera

TABLE II. Hemagglutination-Inhibition Test.

Serum	Antigen					
	Buss.	BSG	Ilhéus	SLE	WN	JE
Bussuquara	160*	10	40	40		
Bat salivary gland	80	160				
Ilhéus	160		1280			
St. Louis encephalitis	10			640		
West Nile	10				320	
Japanese encephalitis	10					160

* 160, titer of serum is 1:160.

TABLE III. Summary of 4 Intracerebral Neutralization Tests.

Serum, immune to — Virus	No. of inoc.	Neutralization indices			
		Virus			
		Buss.	SLE	BSG	Ilhéus
Bussuquara	3	400	1	80	10
St. Louis encephalitis	5	<3	630		
Bat salivary gland	6	10		100,000	
Ilhéus	5	30			1000
Japanese encephalitis	5	5			

from animals bled within 7 to 10 days after a single injection of virus are likely to show a higher degree of specificity than sera obtained after multiple inoculations, some such sera were used for the characterization of Bussuquara virus. From the results (Table II) it can be seen that Bussuquara virus is readily separable from the other agents in the test.

Studies by neutralization test were not as extensive as those done by HI and CF tests, and were limited to viruses and sera with which Bussuquara virus had shown the highest degree of cross reaction in *in vitro* tests. Results of 4 neutralization tests are summarized in Table III.

In these tests, aliquots of a sample of each of 5 immune sera were tested for capacity to protect suckling mice against Bussuquara virus and against each of the homologous viruses with the exception of Japanese encephalitis virus, which was not tested. In addition, Bussuquara virus immune serum was tested against the homologous virus and 3 others. The results of these tests showed clearly that Bussuquara virus is easily distinguishable from SLE, Ilhéus, BSG and Japanese encephalitis viruses. In fact, except for overlap between Bussuquara serum and BSG virus, there was no significant cross reaction.

Summary and conclusions. A virus strain, An 4073, was isolated from blood of a sentinel howler monkey near Belém (Pará), Brazil. This virus yielded a hemagglutinating antigen without difficulty. By means of CF and HI tests with sera from mice immunized with repeated injections, this strain was shown as a member of group B of arthropod-borne viruses. Further studies, in which, in addition, single-injection immune sera and neutralization test were employed, gave evidence that the new agent could be easily distinguished serologically from 14 different group B viruses. Other group B viruses, such as Murray Valley encephalitis and louping ill, with which Bussuquara was not compared are so close to some of those studied that their inclusion in this investigation was considered unnecessary. The limited cross reactions obtained in CF tests between Bussuquara virus and other group B agents indicate a distant relationship. While the negative results may be attributed, in part, to low titers of Bussuquara sera and antigens, more potent systems would still be expected to show the same relative cross reactivity. In view of our results, it is concluded that the strain of virus studied constitutes a new, hitherto unreported arthropod-borne virus belonging in group B. The

virus has been given the name Bussuquara.

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Comparative Response of Normal and Cirrhotic Rats to Intravenously Injected Bacteria.* (24910)

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During study of role of intestinal bacteria in genesis of experimental dietary cirrhosis in rats(1), we noted abscesses in the lung or liver of rats which developed cirrhosis and, by contrast, the absence of such infection in those protected from developing cirrhosis. This suggests that the cirrhotic rats were less resistant to common bacterial invaders. In this communication we report results of a study of antibacterial resistance of these cirrhotic rats.

Method. The experiments were designed to compare ability of normal and cirrhotic rats(1) to clear the circulation of bacteria injected into the portal vein; and (2) to lyse the ingested bacteria. *E. coli,* labelled with I^{131} by method previously reported(2), were washed in saline (0.85%) and then dialyzed against saline at room temperature until almost all radioactivity was removed from supernatant. Assay of the supernatant of the bacterial suspension for radioactivity at various intervals during 48 hours after dialysis, demonstrated that the iodine taken up remained tightly bound. All radioactivity determinations were done with deep well scintillometer. The different bacterial suspensions injected were adjusted by dilution to yield approximately the same concentration of bacteria and were about equal in terms of radioactivity. The viability of these labelled bacteria was tested by subculture every 12 hours. It was estimated from colony counts that all remained viable for duration of experiment.

Normal and cirrhotic† male albino rats of the Wistar strain (wt. 250-300 g) received 10 drops of saturated solution of KI in their drinking water each day for 3 days prior to experiment to saturate the thyroid as well as other tissues with iodine, and thus preclude binding of liberated I^{131}. Through a small midline abdominal incision made under light ether anesthesia, 0.5 ml of a bacterial suspension (10^8 bacteria) of I^{131} labelled *E. coli* was injected into the splenic vein of each rat. The same volume of this suspension was used as standard to measure total radioactivity injected. Smaller injectates gave poorly reproducible curves; larger injectates were rapidly fatal to cirrhotic rats.

Each rat was isolated in individual metabolic cage and all excreta collected. At various intervals after injection the rats were killed by exsanguination. One gram samples of blood, liver, spleen, lung, and urine were planted in various media for cultures of aerobic and anaerobic bacteria. Samples of cirrhotic livers were taken for microscopic confirmation of cirrhosis. Liver, lungs, spleen, kidneys, gastrointestinal tract, thyroid, heart, and a portion of muscle (hamstring) were removed, weighed and homogenized. Appropriate aliquots (2 ml) of the homogenates were then assayed in duplicate for gamma radiation by deep well scintillometer. Remainder of homogenate was then dialyzed at room temperature against running tap water for 24 hours, after which 2 ml aliquots were measured for gamma radioactivity. The total, bound and dialyzable radioactivity

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† Cirrhosis was induced by feeding choline deficient diet for 300 days(1).

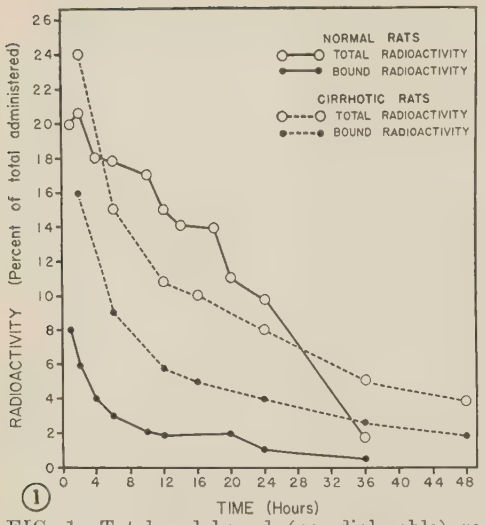


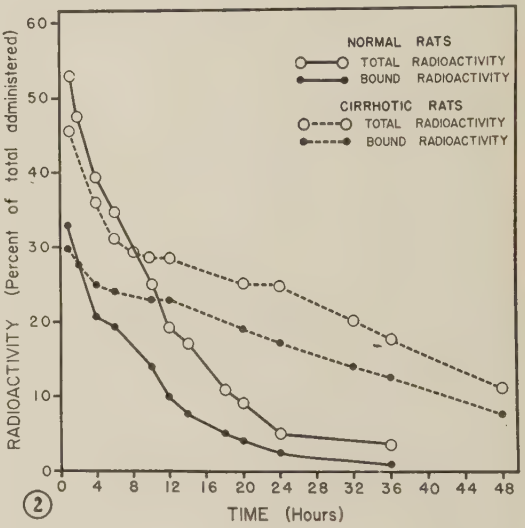
FIG. 1. Total and bound (non-dialyzable) radioactivity in blood of normal and cirrhotic rats at intervals after inj. of I^{131} labelled *E. coli* into the splenic vein.

of blood and each tissue was then calculated,‡ and with the aid of the standard, the results expressed in terms of percent of total radioactivity injected.

That bound and free radioactivity could be taken as a measure of unlysed and lysed bacteria respectively in samples of tissues and fluid was demonstrated by results of experiments in which KI^{131} in saline§ (1 ml) was infused intravenously into normal and cirrhotic rats prefed iodine (KI) as described above. Sixty-five to 78% of the KI^{131} was excreted within 24 hours by both normal and cirrhotic rats, and all radioactivity measured in aliquots of blood and in tissue homogenates was dialyzable. Because nearly all recoverable infusate of I^{131} was rapidly excreted in urine, it is proper to conclude that binding of liberated I^{131} to yield non-dialyzable radioactivity did not occur to any significant extent.

Results. I. *Bacteriological.* A. *Infusion of KI^{131}.* Following administration of KI^{131} to normal rats, all cultures of blood, liver, lung, spleen and urine taken at intervals from normal rats were sterile, while 20% of cultures of blood and liver from cirrhotic rats were positive for *E. coli.*

B. *Infusion of E. coli labelled with I^{131}.* In normal rats *E. coli* were recovered from cultures of spleen to 2 hours after infusion; from blood cultures to 6 hours following infusion; and from cultures of liver and lungs to 12 hours following infusion. In cirrhotic rats, *E. coli* were recovered from blood, liver, and



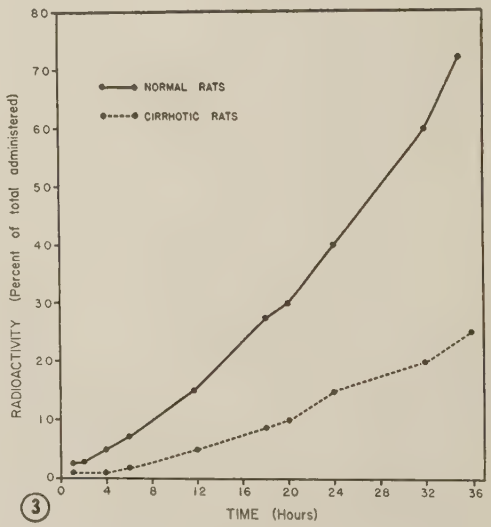


FIG. 2. Total and bound (non-dialyzable) radioactivity in liver of normal and cirrhotic rats at intervals after inj. of I^{131} labelled *E. coli* into splenic vein.

FIG. 3. Radioactivity (ionic) recovered from urine of normal and cirrhotic rats after administration of I^{131} labelled *E. coli* into splenic vein.

‡ Blood volume was estimated to be 10% and skeletal muscle 40% of total body weight.

§ Total radioactivity and iodine content of this solution was approximately equivalent to that of ½ ml of suspension of labelled bacteria.

spleen to 48 hours, and from lung to 32 hours following infusion. In fixed sections of normal livers or lungs bacterial colonies were not seen. On the other hand, colonies of Gram negative bacteria were frequently seen in fixed sections of lungs and in portal areas of fixed sections of liver of cirrhotic rats. Occasionally bacteria formed micro-abscesses. Cultures were sometimes negative when colonies were observed in the sections. In both normal and cirrhotic rats all urine cultures were negative.

II. *Distribution of radioactivity in blood and tissues following injections of I^{131} labelled E. coli into normal and cirrhotic rats.* In *normal* rats, injected bacteria were promptly cleared from the circulation by the liver (Figs. 1 and 2) and lysed since the injected bound radioactivity was rapidly converted to ionic or dialyzable radioactivity; and the latter was promptly excreted in urine (Fig. 3). This rapid disappearance of bacteria from blood, and their uptake by liver, with lysis and release of I^{131} confirmed the bacterial culture data which indicated prompt sterilization of blood and liver.

In *cirrhotic* rats, on the other hand, though uptake of radioactivity by the liver was equal to that in normal rats, the bound or non-dialyzable radioactivity persisted at higher levels than in normal rats for 48 hours (Fig. 3). Only 25% of total radioactivity injected was recovered from urine after 36 hours (Fig. 2). Furthermore, this impairment of bacterial lysis coincided with persistence of positive cultures in blood and liver.

Comment. These data show that the cirrhotic rat clears bacteria from the circulation as well as the normal rat. Persistence of viable bacteria in liver, lung, and spleen in cirrhotic rats suggests that the bactericidal defense in the R.E. system of lung and spleen as well as in liver is damaged. The persistence of viable bacteria in blood suggests that the blood is being reseeded from the persisting foci in the R.E. system.

Summary. Labelled (I^{131}) bacteria infused into portal vein of normal rats were promptly cleared and destroyed with release of ionic I^{131} and its prompt excretion into the urine. In cirrhotic rats the clearance process was normal, but capacity to destroy bacteria was impaired. This resulted in persistence of ingested bacteria and of "bacteria bound" radioactivity in liver, lung, and spleen, and continued reseeding of the blood stream from such foci.

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"Complete" and "Incomplete" Hemagglutin Formation in the Mouse.* (24911)

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The presence of antibodies other than those active in a saline environment has been well documented with human serums following isoimmunization in pregnancy and transfusions. These varieties have usually been categorized as "univalent" or "incomplete" and are demonstrable in solutions of increased viscosity provided by adding serum, albumin, or polyvinylpyrrolidone. Other methods of detecting such antibodies include use of trypsinized erythrocytes and the antiglobulin or Coombs' test. "Incomplete" types of antibodies have also been observed in experimental animals(1, 2,3,4,5,6) but have not been studied in detail. The purpose of this paper is to report the appearance of high titred, "incomplete" hemag-

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TABLE I. Stability of "Complete" and "Incomplete" Antibodies in Anti-Human-Erythrocyte Mouse Serum.

Serum titrations→		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Saline	Original	++++	++++	++	—	—	—	—
	Refrig. 10 mo	"	"	+	—	—	—	—
Albumin (6%)	Original	"	"	++++	++++	+++	++	—
	Refrig. 10 mo	"	"	"	"	++++	+++	—
Trypsin	Refrig. 10 mo	"	"	"	"	+++	—	—
Coombs'	*Idem*	"	"	"	"	++++	+++	—

glutinins for human erythrocytes in response to immunization of the Webster strain of mice. They were demonstrated in 6% human albumin solutions, with trypsinized cells, and by means of a Coombs' reagent made by injecting whole mouse serum into a rabbit.

Materials and methods. To a 5% suspension of washed erythrocytes, was added 0.1 the volume of 1% trypsin followed by incubation at 37°C for 10 minutes. The cells were washed 3 more times in 0.85% saline and a 2% suspension prepared. Three sets of serial 2-fold dilutions of serum were made in 0.2 ml of saline and one in 6% human albumin. One drop from a 1.0 ml pipette (approximately 0.03 ml) of the proper 2% erythrocyte suspension was added to each series. For the saline, albumin and trypsin titrations, the tubes were incubated for 1 hour at room temperature, spun in an angle centrifuge at 1000 rpm for 1 minute and the button examined with a 10X hand lens. Reactions were graded as to intensity. The end-point of each titration was a 2+ agglutination which consisted of readily visible clumps of cells; lesser degrees of clumping were recorded as 1+ but were not included in determining final titre. For the Coombs' titration, approximately 0.03 ml of a 1:100 dilution of the anti-mouse Coombs' reagent was added after the titrations had been incubated for 1 hour and the cells had been washed thoroughly with saline. The tubes were incubated an additional 15 minutes; then centrifuged and examined as above.

Results. In Table I, serum was obtained following intravenous injection of 0.5 ml of packed human group O DCe cells on days 1, 2, 3, 15 and 16 into six 20 g Webster mice. On day 44, the mice received a final injection of 0.5 ml of cells subcutaneously and were bled from the heart 7 days later. When originally tested, this serum showed a saline titre of 1:10,000 and an albumin titre of 1:10,000,000. After 10 months in the refrigerator, it was retested with the results shown below. The remarkable stability of the antibody and the unusually high albumin, trypsin and Coombs' titres following multiple injections of antigen may be noted. Similar responses have also been obtained with sheep erythrocytes.

The result in saline as compared with albumin for a single intraperitoneal dose of varying amounts of human erythrocytes is shown in Table II. With 0.05 ml of cells, both varieties of antibody were produced on the second day after immunization and increased in amount thereafter. The albumin titre was always a few tubes higher than the saline titre. However, if the antigen dose was decreased to 0.005 ml or 0.0005 ml, only the albumin variety of antibody appeared in titres as high as two to the fourteenth power (1:16,384). In other experiments, the "incomplete" titres have varied from 1:512 to 1:4096. Subcutaneous injections of antigen gave similar results.

Discussion. The term "incomplete" anti-

TABLE II. Titer of "Complete" and "Incomplete" Mouse Hemagglutinins after Varying Amounts of Antigen.

	Intraper. dose*					
	.05		.005		.0005	
Day tested	Sal.	Alb.	Sal.	Alb.	Sal.	Alb.
0	0	0	0	0	0	0
2	6†	9	0	4	0	2
4	6	12	0	0	0	0
8	14	16	0	9	0	13
12	10	17	0	9	0	14

* ml packed erythrocytes.

† The number represents a dilution of 2 to the 6th power or 1:64.

body as used in this paper does not necessarily imply that each hemagglutin is physically and chemically separable into different components. It is entirely possible that the albumin, trypsin and Coombs' antibodies are the same as the saline variety but demonstrable only under special conditions. We have used the term in the latter sense until more specific data are available.

The dextran-active mouse hemagglutinin described by Gorer(1,2) in contrast to the one reported herein, was induced by tumor transplants. It was also quite labile on storage and of relatively low titre. Chattergee *et al.*(6) described a mouse, anti-sheep, and anti-rabbit hemagglutinin which was detected in tissue culture by the Coombs' test. Their data, however, were not entirely convincing. Owen(4) discussed a "blocking" and a Coombs' hemagglutinin in the serum of chickens immunized with human erythrocytes; his experimental data were not presented. An unsuccessful attempt to induce "incomplete" antibody in rabbits immunized with human erythrocytes was reported by Foster(7) together with qualitative differences in hemagglutinating versus hemolytic activity during the primary and secondary immune responses. With single injections of human erythrocytes into mice, we have not obtained antibodies with hemolytic properties. However, with sheep erythrocytes as antigen, a hemolysin is regularly induced.

Demonstration of a marked hemagglutin response to primary immunization which is not detected in saline will come as no surprise to those experienced with isoimmunization in pregnancy. One often receives human serums without saline antibodies which, on testing with trypsinized cells, with 20% albumin, or with an anti-globulin serum, are found to contain an Rh agglutinin at a titre of 1:1024 or greater(8). The presence of a similar situation in the mouse suggests that studies in which only the saline or hemolytic variety of antibody has been measured may require reexamination. Thus preliminary experiments with X-radiation of mice indicate that saline and trypsin hemagglutinin formation are inhibited to a greater degree than the albumin or the Coombs' response (Frisch and Davies, unpublished).

Summary. Injection of human, sheep and rabbit erythrocytes into Webster mice results in the appearance of "incomplete" varieties of hemagglutinins detectable in albumin, with trypsinized cells, and by means of a Coombs' reagent. In some animals given small doses of antigen, the saline agglutinin does not appear. At the same time, the "incomplete" ones may be present in high titre and escape notice unless tested for specifically.

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Distribution of Ions in Intestinal Smooth Muscle.* (24912)

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The electrochemical properties of smooth muscle cells are so poorly understood that it is a question whether the relations between ions' distributions and transmembrane potentials is the same as, or merely similar to, the

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relation in other muscle and nerve cells. Bulbring's(1) and Holman's(2) results indicate that potentials of smooth muscle cells do differ from those of skeletal or cardiac in their magnitude and response to drugs and to changes in ionic concentrations in the external environment. Earlier measurements of total amounts of various ions in various smooth muscles were reviewed by Evans(3). When Manery(4) reviewed the subject of electrolyte metabolism little could be added other than that amounts of potassium in smooth muscle were smaller than in striated muscle while amounts of sodium and chloride were considerably greater. Whether larger amounts of sodium and chloride could be accounted for on the basis of a larger extracellular space remained a question. Daniel (5) has shown the difficulty of calculating intracellular ion concentrations on the basis of total amounts present in various smooth muscles and of assumptions which appear to be valid for striated muscle. In the present study an independent estimate of extracellular volume was made by using inulin as an impermeant molecule; total amounts of potassium, sodium, and chloride in pieces of intestinal muscle were determined; and calculations of intracellular ion concentrations were made.

Methods. Segments of ileum and jejunum were removed from young adult cats anesthetized with Nembutal. Cylinders of circular smooth muscle were prepared by stripping away the other intestinal layers(6). Circular muscle segments (0.1 to 0.3 g) to be analyzed for sodium and potassium were prepared quickly, blotted gently and weighed. After drying at 105°C for 24 hr the tissues were weighed again. Weight difference measured total water content. Tissues were ashed at 575°C, the ash dissolved in one drop of 0.1N HCl and diluted to either 10 or 25 ml depending on tissue weight. Sodium and potassium in samples were measured with Baird Associates flame photometer using a lithium internal standard. Standards contained both sodium and potassium in nearly the same ratio as found in muscle unknowns. Samples of blood were obtained by heart puncture. Plasma was obtained by centrifugation, dried, ashed, diluted and analyzed in the same way as muscle. Standards for plasma had appropriate sodium to potassium ratio. Samples for chloride analysis were prepared as above except that the tissues were dissolved in concentrated HNO_3 rather than ashed. The method of Schales and Schales(7) was used. Estimates of extracellular space were made from analyses of inulin content of tissues in equilibrium with 0.5% inulin in Tyrode's solution(8). Inulin used was obtained from Warner-Chilcott as 10% supersaturated solution in 0.5% sodium chloride. The method of Ross and Mokotoff(9) was used for inulin analyses.

Results. Average inulin space for 25 pairs of samples of circular intestinal muscle from 5 cats was 101 cc/kg wet weight. This tissue is apparently more compact than cat or rabbit myometrium, rabbit or guinea pig taenia coli (10) or frog stomach muscle(11). That enough time was allowed for the inulin space to be maximized is shown in Fig. 1. Since the chloride space is larger than the sodium space, intracellular concentration of chloride ions exceeds that of sodium ions. In addition, the sum of intracellular concentrations of potassium, sodium, and chloride ions in the intestinal muscle exceeds their sum in striated muscle(12). This difference is not much changed by using larger estimates of extracellular space in the calculations. Since the osmolality of internal and external solutions must be nearly equal, it must be concluded that there are fewer organic anions in intestional smooth muscle than in striated muscle.

Discussion. The data reported here differ from Daniel and Daniel's(10) results not in total amounts of potassium, sodium and chloride present in smooth muscle but rather in volume of extracellular space as approximated by an inulin space.

The chloride space cannot be used as a valid approximation of extracellular space because it exceeds the sodium space and thus calculations with it yield negative intracellular sodium ion concentration. Use of sodium space is not warranted since sodium is known to be contained in other muscle cells. Since the extracellular solution contains relatively more water and very little potassium, the calculated intracellular potassium concentrations

TABLE I. Distribution of Ions and Water in Cat Intestinal Circular Muscle.

	Intestinal muscle, meq/kg wet wt	Thigh muscle, meq/kg wet wt	Plasma, meq/l	Intestinal muscle, meq/kg cell H_2O
Potassium	77.0 ± 2.5 (27)	87.2 ± 1.8 (4)	3.7 ± .3 (8)	108 ± 6
Sodium	61.3 ± 1.5 (27)	35.2 ± 2.1 (4)	141 ± 3 (8)	65 ± 5
Chloride	66.6 ± 2.2 (13)		122 ± 2 (8)	77 ± 5
H_2O, g/kg	810 ± 2 (57)	804 ± 4 (4)	929 ± 2 (8)	
	cc/kg wet wt			
Inulin space	101 ± 17 (25)			
Chloride "	544			
Sodium "	432			

Numbers in parentheses indicate No. of measurements contributing mean values.

rise quickly to levels which indicate binding as larger values for extracellular space are assumed (10).

Conway (12) reviewed the methods and sources of error associated with estimation of extracellular space. He concluded that the inulin technic was as valid as any currently available. Calculations of intracellular concentrations based on an inulin space yield values for intestinal smooth muscle which are different than those for striated muscle.

Using intracellular concentration of potassium based on the inulin space a maximum diffusion potential of 84 mV may be calculated. Maximum values of the resting potential recorded in a parallel study to be reported elsewhere were less than 80 mV. Thus it would appear that in intestinal smooth muscle the potassium ion gradient is large enough to support the hypothesis that the resting membrane potential is essentially a diffusion potential dominated by the potassium gradient.

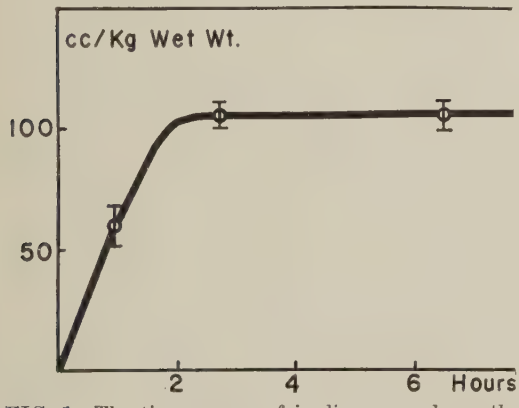

FIG. 1. The time course of inulin space shows the approach to equilibrium occurs after about 2 hr. Circles represent means of from 8 to 14 values and vertical lines represent stand. error of means.

Summary. Cat intestinal smooth muscle had an inulin space of 101 ± 17 cc/kg wet weight. Total water, potassium, sodium and chloride were determined and intracellular concentrations of potassium, sodium and chloride were calculated to be 108 ± 6, 65 ± 5, and 77 ± 5 meq/kg cell H_2O; respectively.

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Antibody Response of Adult Mice to Virus of Foot-and-Mouth Disease. (24913)

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An immune response to foot-and-mouth disease virus (FMDV) in adult mice was reported by Skinner *et al.*(1), who observed that offspring of mother mice inoculated during pregnancy were resistant to virus challenge. Although suckling mice are susceptible, adult mice are usually considered resistant. In studies, normal adult mice were unaffected clinically after inoculation with FMDV unless some process of virus selection or host treatment was utilized(2-4). Because of advantages of adult mice, the present experiments study neutralizing antibody response of such animals to FMDV to learn when antibody appears and when highest antibody level is reached.

Materials and methods. Adult female Rockefeller H strain mice, 120 days old at time of inoculation, were used for antibody production. Litters of 10 suckling mice of same strain, 5-10 days of age, were used in neutralization tests for detection of antibody produced in adult mice(1). Foot-and-mouth disease *virus,* type A, strain 119, produced in Roux flask cultures of bovine kidney cells(5) was the stock virus preparation. Nutrient fluid from cultures of infected cells was centrifuged in Spinco Model L centrifuge (No. 30 rotor) at 10,000 rpm for 30 minutes to remove cellular debris. The supernatant fluid containing the virus was stored in sealed ampoules at –40°C. This material was considered as undiluted stock virus. Dilutions of stock virus were prepared in tryptose phosphate broth (pH 7.4). Two experiments were performed, the first with 40 and the second with 38 adult mice. Mice were inoculated intraperitoneally (IP) with 0.1 ml of undiluted stock FMDV. At intervals of approximately 24 hours after inoculation in first experiment, blood was obtained and pooled from 2 randomly selected mice by incision in axillary region after ether anesthesia. In the second experiment, blood was obtained at intervals of approximately 24 hours for first 2 weeks and 7-day intervals thereafter. Serums collected from pooled blood were placed in rubber-capped vaccine vials and stored at –40°C until all serums from a given experiment were available for testing at the same time. Antibody content of adult mouse serums was studied by neutralization tests in suckling mice, 5-10 days of age, using dilutions of FMDV mixed with constant amount of serum previously heated at 56°C for 30 minutes. Beginning with 1:5 dilution of stock virus, with a titer of approximately $10^{6.6}$ LD_{50}/ml in suckling mice, serial 10-fold dilutions were prepared; and to 0.5 ml of each dilution, 0.5 ml of serum was added. Final virus dilutions were 10^{-1} through 10^{-7}. To induce death in all mice in at least the 10^{-1} dilution so that calculations of neutralizing indices could be made, serums collected to approximately 10 days postinoculation were diluted initially 1:20. Serums collected subsequently were diluted initially 1:1000. Allowance for these dilution factors was made in calculations for various serums by expressing LD_{50} neutralization indices on the basis of ml of undiluted serum. Virus-test serum mixtures and controls consisting of virus-normal mouse serum and virus broth mixtures were incubated in water bath at 37°C for 30 minutes, after which each mixture was inoculated into a litter of 10 suckling mice. Each mouse was inoculated IP with 0.05 ml. The mice were observed daily for 10 days after inoculation. Viral end points were determined by method of Reed and Muench(6).

Results of neutralization tests are summarized in Tables I and II. In Exp. 1, observations were made periodically from 3rd through 32nd day after inoculation, and in Exp. 2,

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from 1st through 84th day after inoculation. Serums collected on first and second day after inoculation had no demonstrable antibody. On 3rd day after inoculation, however, Exp. 1 sample had a virus-neutralizing index of 2.48 logs/ml of undiluted serum, and Exp. 2 sample had a virus-neutralizing index of 3.61 logs/ml of undiluted serum. A gradual increase in neutralization indices of serums in each experiment occurred thereafter until they leveled off at between $10^{6.0}$ and $10^{7.0}$ LD_{50}/ml, approximately 20 days after mice had been inoculated.

Discussion. Antibody response of adult mice inoculated with FMDV indicated that after IP inoculation of 0.1 ml of tissue culture fluid containing approximately $10^{6.6}$ suckling mouse LD_{50} of FMD/ml, adult mice produced neutralizing antibody detectable within 3 days after inoculation. In general, the antibody reached a maximum between 20 and 30 days after inoculation and remained at high level throughout the 84 days of Exp. 2. An exception can be found in neutralizing indices of serums collected on 56th and 63rd days of Exp. 2 (Table II), which were greater than those of serums collected on 21st and 28th days. It was not our purpose to determine persistence of antibody in adult mice. The experiments indicate, however, that a significant level of neutralizing antibody persists for at least 3 months after inoculation.

TABLE I. Neutralizing Capacities of Serums Collected at Varying Intervals from Adult Mice Inoculated with Foot-and-Mouth Disease Virus.

Exp. I

Serum sample D.P.I.*	LD_{50} neutral. index uncorrected for ser. dil.	Final serum dilution (Log, base 10)	LD_{50} neutral. index/ml of undil. ser.
0	.15†	−1.6	
3	.88	"	2.48
4	2.14	"	3.74
5	2.60	"	4.20
6	1.10	−3.3	4.40
7	1.93	"	5.23
10	1.31	"	4.61
11	1.79	"	5.09
12	2.50	"	5.80
14	1.87	"	5.17
18	2.66	"	5.96
20	3.41	"	6.71
24	3.26	"	6.56
26	2.79	"	6.09
31	2.94	"	6.24
32	2.00	"	5.30

* D.P.I. = Days postinoculation.
† Not considered as evidence of neutralization.

TABLE II. Neutralizing Capacities of Serums Collected at Varying Intervals from Adult Mice Inoculated with Foot-and-Mouth Disease Virus.

Exp. II

Serum sample D.P.I.*	LD_{50} neutral. index uncorrected for ser. dil.	Final serum dilution (Log, base 10)	LD_{50} neutral. index/ml of undil. ser.
0	.09†	−1.6	
1	.00†	"	
2	.30†	"	
3	2.01	"	3.61
4	2.90	"	4.50
8	1.88	"	3.48
10	1.09	−3.3	4.39
11	.90	"	4.20
14	1.44	"	4.74
21	2.71	"	6.01
28	2.85	"	6.15
35	2.24	"	5.54
42	2.33	"	5.56
56	2.92	"	6.22
63	3.07	"	6.37
70	.64	"	3.94
78	1.79	"	5.09
84	1.49	"	4.74

* D.P.I. = Days postinoculation.
† Not considered as evidence of neutralization.

Seventy-eight adult mice were inoculated with FMDV. In addition, 24 adults were inoculated in a preliminary experiment not included. In this group of 102 mice plus approximately 50 adult mice used as controls in other experiments now in progress, no normal adult mouse died because of infection with FMDV or revealed any other symptoms of infection. This lack of signs of illness in adult mice is in accordance with previous observations(2-5).

Summary. Normal adult female mice were inoculated with the virus of foot-and-mouth disease, and serums obtained from these animals at intervals up to 3 months after inoculation were tested for their neutralizing capacities. Neutralizing antibody was detected as early as 3 days after inoculation and increased gradually until a maximum was reached at 20-30 days. The antibody persisted at a high level throughout remainder of 84-day period.

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Direct Evidence for Fatty Acid Mobilization in Response to Growth Hormone Administration in Rat.* (24914)

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It has long been known that anterior pituitary gland extracts and purified growth hormone preparations, when administered to experimental animals, increase the concentration of lipids in plasma and the liver while decreasing amount of total body fat (see 1 and 2 for review). More recently, it has been demonstrated that small doses of growth hormone cause a rapid increase in the plasma nonesterified fatty acid (NEFA) concentration in man(3), rhesus monkey(4) and rat (5). These effects of growth hormone on levels of circulating lipid have been interpreted in terms of an increase in mobilization of lipids from adipose tissue but direct evidence for such an interpretation has been lacking. The following experiments were performed to establish the effect of prior growth hormone administration on release of NEFA from isolated adipose tissue *in vitro*.

Material and methods. Male rats of the Charles River strain weighing between 100 and 150 g were used. Hypophysectomies were performed 4 weeks prior to experiment. All animals were fasted 24 hours before autopsy. In the "acute" experiments a single intraperitoneal injection of bovine growth hormone† (3 mg) was administered three hours before sacrifice. In the "chronic" experiments bovine growth hormone (3 mg per rat per day) was given intraperitoneally for 3 days and the rats sacrificed on the day following last injection. Control animals were injected with equal volumes of normal saline. All animals were anesthetized with 30 mg sodium pentobarbital given intraperitoneally. The epididymal fat bodies were removed and incubated individually (2 for each animal) in a system previously described(6). NEFA released into the incubating medium was determined by the method of Dole(7) and expressed as μeq. NEFA released per g wet weight of tissue per hour. Blood glucose concentrations were determined by the method of Nelson(8) and plasma NEFA concentrations by the method of Dole(7).

Results. Hypophysectomy reduced release of NEFA from adipose tissue when compared with normal control animals fasted for the same length of time (Table I). Administration of a single 3 mg dose of growth hormone to hypophysectomized animals 3 hours before sacrifice significantly ($P<.01$) increased NEFA release from adipose tissue *in vitro* (Table I). No difference in blood glucose

TABLE I. Effect of Hypophysectomy and Growth Hormone Treatment on NEFA Release by Adipose Tissue. Ten animals/series; 2 observations/animal.

Rats	NEFA release, μeq/g/hr
Normal control	5.79 ± .44‡
Hypox. + saline	.90 ± .16
" + G.H. (acute)*	2.30 ± .37
" + " (chronic)†	3.40 ± .66

* 3 mg/rat 3 hr before sacrifice.
† 3 mg/rat/day for 3 days.
‡ Mean ± S.E.

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† The bovine growth hormone was a gift of Endocrinology Study Section, N.I.H.

concentration between these animals and their saline-injected controls was observed.

Chronic growth hormone administration (3 mg/rat/day for 3 days) to hypophysectomized rats also increased rate of NEFA release ($P<.01$) from their epididymal fat bodies (Table I). Under these circumstances plasma NEFA and blood glucose concentrations were unchanged by the growth hormone treatment.

Discussion. The results of this study clearly indicate that hypophysectomy inhibits and growth hormone stimulates release of NEFA from adipose tissue and validate the conclusion that the increase in plasma NEFA concentration observed following growth hormone administration signifies increased NEFA mobilization from fat stores. The mode of action of growth hormone in this regard, however, is not clear. That growth hormone does not have a direct lipolytic action on adipose tissue has been suggested by the experiments of White and Engel(9) in which growth hormone was added to adipose tissue *in vitro*.

It has been suggested(10) that the regulation of NEFA mobilization is mediated by availability of glucose for cellular oxidation. If the action of growth hormone is explicable on this basis, *i.e.* by inhibiting glucose oxidation, it was not reflected by alterations in blood glucose concentrations as measured in the present experiments.

Summary. The release of nonesterified fatty acids (NEFA) from epididymal fat bodies removed from normal, hypophysectomized and hypophysectomized growth hormone treated rats has been investigated. Hypophysectomy inhibits and growth hormone administration stimulates NEFA release from adipose tissue *in vitro*. These findings are taken as direct evidence for the concept that growth hormone treatment causes NEFA mobilization from fat stores.

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Quantitative Determination of Infectious Units of Measles Virus by Counts of Immunofluorescent Foci.* (24915)

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Enumeration of infectious animal virus units depends either upon determination of dilution endpoint or upon counting foci of virus growth(1). The latter approach, modeled after bacterial colony count technic, has been utilized to count proliferative and necrotic pocks on animal skin and cornea, and on chorioallantois of embryonic chick. It is now widely employed for counting plaques in cultures of animal cells(2). Many viruses that multiply in animal cells do not form plaques. Demonstration and enumeration of

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foci of virus antigen in such cells can perform the same function as plaque counts. Dulbecco and Vogt(3) suggested that the fluorescent antibody technic would serve this purpose. Noyes(4) counted fluorescent foci obtained with West Nile virus by a method that enabled him to compare relative concentrations of virus; and Hotchin(5) reported that methyl cellulose, which does not fluoresce, could be used as substitute for agar in an overlay designed to prevent secondary plaque formation. We found that counts of fluorescent foci can be used to measure amount of infectious virus in suspensions of a measles strain that fails to form well-defined plaques in the cell-virus system. The technic should be useful for quantitative investigation of other viruses in cells that support virus growth but in which plaques are not recognized.

Materials and methods. The Edmonston strain of measles virus(6)‡ was employed. The virus had undergone 26 passages in human kidney cells, 8 passages in human amnion cells, and 29 passages in human epidermoid carcinoma No. 2§ (H. Ep.-2) cells. In H. Ep.-2 cells under fluid, the virus induces formation of giant cells and of long cellular processes, and also causes necrosis, but as mentioned above, countable plaques were not observed under agar overlays. H. Ep.-2 cells were grown on 12 mm round coverslips in 60 mm petri plates, 6 coverslips/plate. Four drops (approximately 0.2 ml) of a suspension containing 100,000 H. Ep.-2 cells/ml were placed on each coverslip and allowed to attach to the glass for approximately 3 hours at room temperature. The cells were then flooded with 4 ml of nutrient fluid consisting of 80% Eagle's basal medium,‖ 15% pooled horse serum, and 5% calf serum.‖ Penicillin (100 units/ml), streptomycin (100 μg/ml) and nystatin (Mycostatin, Squibb, 100 units/ml) had been added to the fluid. The pH was then adjusted to 7.5 with $NaHCO_3$. The petri plates were put unsealed into standard desiccator together with a lighted candle. The desiccator was tightly closed, sealed, and incubated at approximately 37°C. After 3 days, good sheets of cells had usually formed, and the coverslips were transferred to dry petri dishes 0-2 days later. For virus assays, cell sheets were inoculated with 0.05 ml of appropriate virus dilution. Each virus dilution was tested on 4 or more coverslips. The virus was permitted to adsorb for 2 hours at room temperature before an overlay was added. The overlay, consisting of 2% methyl cellulose and 20% horse serum in Eagle's basal medium, was prepared as follows: the methyl cellulose (4000 centipoises) was repeatedly washed with absolute ethanol and with ether, then air dried. Two g of the powder were suspended in 40 ml of boiling distilled water, autoclaved, and cooled to approximately 40°C. Forty ml of double strength Eagle's basal medium with antibiotics and 20 ml of horse serum were then added. The suspension was mixed well and stored at 4°C as a viscous sol. Inoculated cells in plates were reincubated for 3-6 days in the candle desiccator at 37°C. At this temperature the viscous methyl cellulose almost gels. After 3-6 days, plates were refrigerated at 4°C for approximately one hour, which reliquefies the methyl cellulose. Although the overlays were still viscous, they could then be decanted, following which the coverslips were washed repeatedly with chilled, phosphate-buffered saline. The cells were fixed with acetone for 10 minutes at room temperature, and prepared for immunofluorescent microscopy by indirect technic(7,8). Convalescent-phase serum from measles patients was used as antimeasles antibody. Fluorescein-labeled antihuman globulin horse pseudoglobulin had previously been absorbed twice with mouse liver powder to remove nonspecific staining properties(7,9). Controls consisted of infected cells treated with acute-phase serum from measles patients, and uninfected cells treated with convalescent-phase serum; both sets were then reacted with labeled antihuman pseudoglobulin. Coverslips were mounted in phosphate buffered glycerol (pH 7.0) on glass slides. All counts were done on coded specimens. They were made with the aid of eyepiece grid and mechanical

‡ Dr. John Enders, Children's Hospital, Boston, kindly furnished the virus.

§ Dr. Alice Moore, Sloan-Kettering Inst. for Cancer Research, N. Y., kindly supplied H.Ep.-2 cells.

‖ Microbiological Assoc.

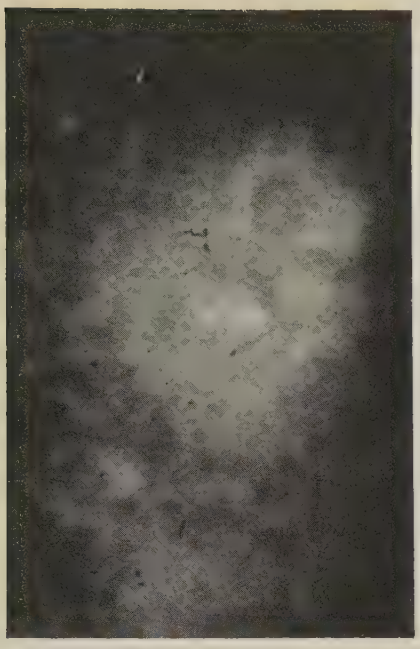

FIG. 1. Immunofluorescent focus of measles antigen in H.Ep.-2 cells after 5 days of development under methyl cellulose overlay. 530×.

stage under low power (100X) of the fluorescence microscope, similar to the one previously described(10). Photomicrographs were taken with camera coupled to Leitz Micro-Ibso attachment. Anscochrome film was exposed 8 minutes and processed as recommended by the manufacturer, although it was found advantageous to develop at ASA 125. Black and white reproductions were prepared from color slides. Concurrent infectivity titrations of the virus were carried out in 16 x 125 mm screw cap tubes containing monolayers of H. Ep.-2 cells. Our experiments involved 24 tubes/dilution; each tube received 0.05 ml of inoculum. Tubes were incubated in stationary racks at 37°C and the cells inspected for cytopathogenic changes for 14 days following inoculation. Fluid changes were carefully performed to prevent cross-contamination; separate pipettes were used for each tube.

Results. Fluorescent foci appear in H. Ep.-2 cells infected with Edmonston strain of measles virus as early as 12 hours after inoculation(11), and in the absence of an overlay a few foci consisting of single cells or small groups of cells can regularly be distinguished one day after inoculation. Multicellular foci appear within 3 days, but single-cell or small foci are also present at this time. This polymorphism is probably the result of secondary spread of infection.

Most fluorescent foci seen after 3 days incubation under methyl cellulose consisted of 1-2 cells. Five day foci were multicellular, approximately circular, relatively uniform in size, and were readily countable at 100X magnification (Fig. 1). They appeared to be randomly distributed on coverslips.

Five aliquots of stock measles virus suspension were assayed separately on different days and the results compared. Three assays were done by means of fluorescent focus counts in which methyl cellulose overlays were employed, while the fourth and fifth were infectivity titration in tubes. H. Ep.-2 cells were used for all 5 experiments, and the volume of inoculum was 0.05 ml for each coverslip preparation or tube. Inocula were stored sealed in dry ice box until diluted for use.

A fluorescent focus count assay that was read after 5 days of incubation will be presented first (Table I, solid circles in Fig. 2). There was good correlation between relative amount of virus in the inoculum and corresponding mean count. In the range of 125-8.3 mean counts/dilution, 2-fold dilutions of virus yielded slightly greater than 2-fold de-

TABLE I. Counts of Immunofluorescent Foci on Coverslips 5 Days after Inoculation of 2-Fold Dilutions of An Aliquot of Stock Measles Virus.

	Immunofluorescent focus counts			
Dil. of virus	Each coverslip	Mean ± S.D. of mean	Variance	Fold decrement
1:25	173, 114, 84, 135, 139, 119, 117, 119	125.0 ± 9.0	648	
1:50	17, 56, 55, 39, 59, 58, 47, 53	48.0 ± 5.0	200	2.6
1:100	23, 17, 20, 22, 19, 18	19.8 ± .94	5.37	2.4
1:200	8, 12, 11, 1, 7, 11	8.3 ± 1.67	16.7	2.4
1:400	5, 3, 1, 10, 4, 7	4.7 ± 1.23	9.07	1.8

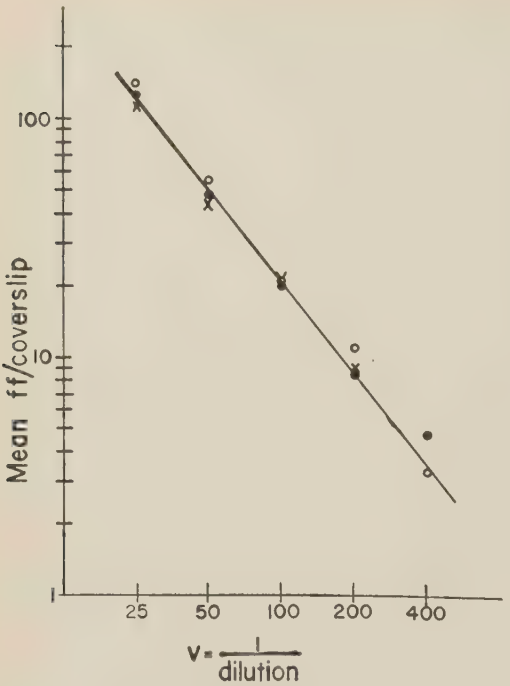

FIG. 2. Relationship between virus in inoculum (V) and counts of immunofluorescent foci (ff) on coverslips 3, 5, and 6 days after inoculation of 3 different aliquots of stock measles virus on different days. ○ = 3 day foci; ● = 5 day foci; and × = 6 day foci.

crements in number of foci counted.

The 5 day counts were confirmed by fluorescent focus count assays of 2 other aliquots of stock virus suspension (Fig. 2). Average counts at end of 3 days (open circles) and 6 days (crosses) incubation are plotted, together with data from the assay just discussed. On the whole the 3 assays agree. Since the counts were made after differing incubation periods, these results, in addition to indicating the degree to which the procedure is reproducible, demonstrate that formation of secondary foci probably occurs to a negligible extent under the overlay, between the time that foci first appear (3 days) and the time that multicellular foci have formed (5 and 6 days).

To estimate the virus content of the inoculum by an independent method, half-log dilutions respectively of a portion of the aliquot used for the 5 day counts, and of another aliquot of the same stock virus suspension, were inoculated into tubes of H. Ep.-2 cells, 24 tubes/dilution. The percentage of tubes exhibiting cytopathogenicity (Table II) fits the Poisson distribution for the hypothesis that one infectious particle can initiate infection. Such an all-or-none method as tube titration is inherently less precise than a plaque or focus counting technic unless more than 24 tubes/dilution are employed. When calculations are based on mean fluorescent focus counts in the range of 20 foci/coverslip or less, there appeared to be approximately 3 to 4 times as many fluorescent foci/unit volume of stock virus suspension than would be expected from the results of tube titration. While the conclusion that 3-4 fluorescent focus units are equal to a unit capable of giving rise to cytopathogenicity is not warranted from this experiment because of technical differences in the 2 types of assays, it is a possibility that deserves further study.

Discussion. The procedure described has marked advantages for several types of investigation. First, it can be employed, as in this study, to determine quantitatively the amount

TABLE II. Distribution of Endpoint Cytopathogenic Changes in Tubes Containing H. Ep.-2 Cell Monolayers 14 Days after Inoculation of $10^{-0.5}$-fold Dilutions of An Aliquot of Stock Measles Virus.

Dil. of virus (−log)	No. of tubes		% positive tubes	
	Inoculated	Positive*	Observed	Expected†
2.0	24	22	92	99
2.5	"	19	79	76
3.0	"	5	21	36
3.5	"	3	13	13
4.0	"	2	8	6
4.5	"	0	0	1
5.0	"	0	0	<1
1.5	"	24	100	99
2.0	"	24	100	99
2.5	"	21	88	86
3.0	"	12	50	47
3.5	"	5	21	18
4.0	"	1	4	6

* Tubes showing cytopathogenicity were scored "positive."

† On assumption that one infectious particle is capable of initiating infection, and that such particles are distributed by the Poisson distribution, the expected number of particles/tube was taken as arithmetic mean of Poisson estimates for dilutions in which both "positive" and "negative" tubes occurred. Expected percentages of "positive" tubes were calculated from expected number of particles/tube.

of virus that will be productive of infection in a cell system that does not form plaques under conditions employed. Second, the method might be useful for assay of non-plaque forming variants in a cell-virus system in which plaques are formed. Third, the technic might be practicable for study of latently infected cells. Use of the method as assay procedure to detect foci of incomplete virus multiplication, in which virus antigen is produced, might be listed in any of these 3 categories.

The procedure is time-consuming and the technic is demanding, so that assay by fluorescent focus counts cannot be recommended as convenient. The chief practical difficulties seem to occur at the time the infected coverslips are prepared for fluorescence microscopy. Sometimes it is difficult to wash methyl cellulose completely from the infected coverslips, and if this is the case, the reaction of virus antigen with antibody is probably blocked, and quantitative results cannot be obtained. A second practical difficulty that has been encountered is due to the variability of different batches of fluorescein-labeled antibody with respect to antigen-antibody combination. With respect to another potential source of error, it is essential that a measured volume of virus suspension be adsorbed to a cell sheet having defined limits. Confinement of an inoculum to the surface of a coverslip culture can be achieved by transfer of coverslip to a dry surface before addition of inoculum.

The conclusion that the methyl cellulose overlay was effective in preventing formation of secondary foci is supported by other evidence in addition to the data (Fig. 2) already presented. Foci spread concentrically from the few cells observed at end of third day of incubation. The regular, circular multicellular areas containing virus antigen that had formed at the end of 5 days incubation were relatively uniform in size. Furthermore, foci were apparently distributed at random on the coverslips; and finally, on some coverslips, there was only one focus.

Counts were approximately proportional to amount of virus added. This result is consistent with the hypothesis that each focus is the consequence of infection with a single infectious particle of measles virus. Additional evidence for a one particle hypothesis was obtained by tube infectivity titrations since the proportion of tubes exhibiting cytopathogenic changes closely followed the expected Poisson distribution.

Summary. 1) The number of infectious units of measles virus in a suspension were assayed by counting microscopically the number of immunofluorescent foci in infected coverslip tissue cultures, after they were incubated under methyl cellulose overlay. The overlay prevents secondary spread of virus and is removed before the infected cell sheets are treated with antimeasles serum and fluorescein-labeled antiglobulin for detection of virus antigen by the indirect fluorescent antibody procedures. 2) Aliquots of stock virus suspension were serially diluted and assayed separately by this procedure. Results of independent assays agreed well. Ratios between fluorescent focus counts on successive dilutions approximated the reciprocal of dilution factor and there appeared to be a linear relation between amount of virus inoculated and count. In comparison with a virus titration in monolayers of same cell in tubes read for endpoint cytopathogenicity, fluorescent focus count assay of same stock virus had the same advantage as plaque technic. Data were consistent with the hypothesis that one infectious particle of measles virus is sufficient to initiate infection. 3) The method seems useful for investigation of latent infection, incomplete virus multiplication, non-plaque-forming variants of plaque-forming viruses, or other cell-virus systems in which virus antigen is produced but plaques are absent.

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Distribution Kinetics of Intravenous Epinephrine. (24916)

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Epinephrine given intravenously rapidly disappears from plasma(1). Guyton and Gillespie found that infusion of .0025 mg/kg/min into a dog sustained blood pressure at 200 mm Hg(2). Using constancy of pressure as criterion they calculated that rate of destruction was directly proportional to quantity in the body; the amount destroyed/minute being .631 times that quantity. The following studies were carried out to analyze, by means of fluorometrically determined plasma levels, distribution kinetics and disappearance rate of intravenously injected epinephrine.

Methods. Mongrel dogs of both sexes weighing 8 to 20 kg were anesthetized with intravenous pentobarbital. Six were given rapidly into femoral vein a single injection of 40 μg epinephrine/kg as l-epinephrine bitartrate. Five were similarly injected, then immediately given a constant intravenous infusion of epinephrine to 75 minutes duration. Blood pressures were recorded continuously from a cannula in femoral artery. Heparinized blood was drawn from opposite femoral artery immediately prior to, in some instances during, and at frequent intervals following administration of epinephrine. Plasma epinephrine contents were determined fluorometrically by modification of the method of Lund(1,3).

Results. Immediately following injection of 40 μg epinephrine/kg blood pressures rose to 300-350/200-250 mm Hg, remained for approximately one minute, fell during the next minute to 160-180/100-120 mm Hg, then slowly returned toward pre-injection levels over subsequent 4 minutes. During continuous infusions, blood pressures were maintained at about 200/150 mm Hg, then at cessation fell to 50-70/30-50 mm Hg within 2 minutes.

When epinephrine is given rapidly intravenously, arterial plasma levels reach a peak within several seconds and fall to pre-injection values within 4 to 6 minutes (Fig. 1). Mean half-time for disappearance from the plasma compartment of epinephrine, which is in excess of pre-injection level, is 23 seconds. Mean calculated disappearance rate constant (k_1)† is −1.77. By extrapolation to zero time the calculated volume of distribution of injected epinephrine averages 45 ml/kg. This approaches the plasma volume of the dog(4).

Five experiments were carried out in which epinephrine was infused at a constant rate following rapid injection of 40 μg/kg. When given at a rate of 11 or 12 μg/kg/min, plasma levels continued to rise at 2 μg/l/min. When administered at a rate of 6 or 7 μg/kg/min more nearly constant levels of about 100 μg/l

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† k_1 (disappearance rate constant for rapid injection) and k_3 (disappearance rate constant for slow phase after prolonged infusion) $= \frac{2.303}{T_1 - T_2} \times \log \frac{C_{T_1} - C_0}{C_{T_2} - C_0}$; where C_0 is pre-injection plasma epinephrine concentration and C_{T_1} and C_{T_2} are concentrations at T_1 and T_2 minutes, respectively. k_2 (disappearance rate constant for rapid phase after prolonged infusion) $= \frac{2.303}{T_1 - T_2} \times \log \frac{C_{T_1} - C_{Eq}}{C_{T_2} - C_{Eq}}$; where C_{Eq} is plasma epinephrine concentration (21 μg/l) at beginning of slow disappearance phase. Regression lines calculated by method of least squares.

were present after 30 minutes. Six μg/kg/min is somewhat greater than the disappearance rate of epinephrine.

In these experiments the plasma epinephrine level was followed after cessation of infusion. It rapidly fell within 4 or 5 minutes to approximately 5 times average pre-injection concentration (Fig. 2). The calculated half-time of 38 seconds for disappearance of epinephrine, which is in excess of this new "equilibrium" level of about 21 μg/1, is slightly greater than that found following single injection. Mean calculated disappearance rate constant $(k_2)^{\dagger}$ after cessation of continuous infusion was –1.08.

Occurrence of this new "equilibrium" level by 5 minutes after termination of constant infusion means that the rapid disappearance rate has ceased. Therefore, in one experiment plasma epinephrine level was followed for one hour beginning 5 minutes after constant infusion was discontinued (Fig. 3). Plasma concentration changed very slowly; half-time for decline of plasma epinephrine in excess of pre-injection level in this period was 23 minutes and disappearance rate constant $(k_3)^{\dagger}$ was –0.030. During this hour 8.64 μg appeared in urine, while during the hour of infusion 2.24 μg were excreted. These were respectively 0.06 and 0.01% of total given.

Discussion. Rate of disappearance of epinephrine from plasma following single injection or immediately following termination of constant infusion is very rapid. Radiosulfate has a half-time of 4 minutes(5). The speed with which potassium leaves the circulation approaches that of epinephrine(6). Weil-Malherbe and Bone found physiological amounts of epinephrine in platelets(7). While our methods did not necessarily include epinephrine in platelets, this substance is not readily taken up by platelets *in vivo*(8). Storage by platelets therefore is not apt to accelerate the rate of disappearance from plasma. Epinephrine is almost completely bound to plasma proteins, especially albumin(9). However, such binding does not appear to retard seriously the disappearance of epinephrine from plasma.

Utilization or degradation of epinephrine would give an accelerated rate of disappearance compared to substances not so metabolized. On the basis of arterial-venous differences in human plasma, rate of utilization has been calculated to be 0.033 μg/kg/min(10). Rate of degradation by certain organs is high. Lund calculated that rabbit liver destroys up to 10 mg/kg/min, while the intact dog eliminated 10 μg/kg/min(11). He postulated that epinephrine could 1) be destroyed by oxidative deamination, 2) diffuse into tissues, 3) be oxidized through the adrenochrome stage, and 4) be excreted by the kidneys. Although degradation by amine oxidase has been considered important, Corne and Graham found that inhibition of this enzyme in the cat with 1-isonicotinyl-2-isopropyl hydrazine did not alter pressor response to infused epinephrine (12). Inhibition did raise urinary excretion of free epinephrine from 2.5 to 5.2%. As methylation to metanephrine probably precedes oxidative deamination by monamine oxidase, inhibition of this enzyme does not lead to a significant increase in unmetabolized epinephrine in urine(13).

Circulating epinephrine is removed by hind leg, liver and kidney(14). It is not known how much is destroyed by or diffuses into these tissues. Little enters or is metabolized by red blood cells(15). No gross change in disappearance rate is found when circulation is shut off to major organs(16). Similarly evisceration, hepatectomy, and nephrectomy do not markedly alter the rate(15). Only a small fraction of infused epinephrine appears in urine, although slightly larger percentages have been reported(17,18).

Rate of removal of epinephrine following single injection of 20 μg/kg has been calculated to be 3 to 5 μg/kg/min(15). This rate is similar to that reported here. As amount destroyed is proportional to quantity present, a more general description, based on the rate constant derived, is that 0.83 times the amount present in plasma disappears each minute. The disappearance rate immediately following cessation of constant infusion is slightly less than that after single injection. Jones and Blake utilizing shorter infusion periods found a similar rate during this time (14). Their calculated distribution volume of epinephrine was at least twice plasma vol-

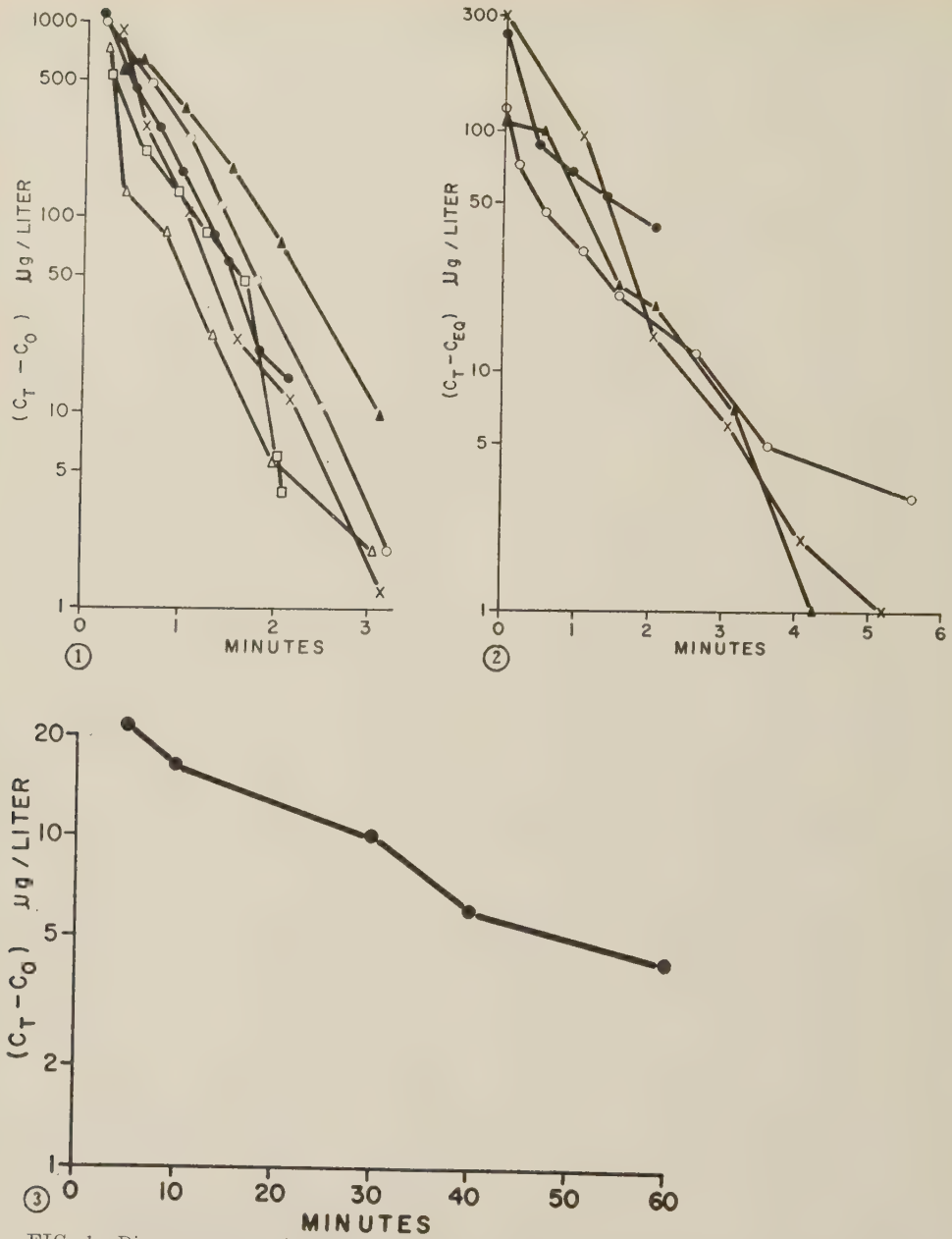

FIG. 1. Disappearance of epinephrine from dog's arterial plasma following rapid intrav. administration of 40 µg/kg. Difference between arterial plasma concentration of epinephrine at each time (C_T) and pre-inj. level (C_0) is plotted logarithmically against time. Lines connect logarithms of actual values obtained from each dog. Calculated regression line for entire group is $Y = 2.964 - 0.770\ X$. Individual slopes differ significantly from mean regression coefficient.

FIG. 2. Disappearance of epinephrine from arterial plasma immediately following discontinuance of constant infusions given at rate of 12.51 µg/kg/min. for 35 min. (●); 11.22 µg for 35 min. (×); 7.34 µg for 60 min. (○), and 6.38 µg for 75 min. (▲). Difference between arterial plasma concentration of epinephrine at each time (C_T) and "equilibrium" concentration (C_{Eq}) reached at 4 or 5 min. is plotted logarithmically against time. Lines connect logarithms of actual values. Calculated regression line for entire group is $Y = 2.169 - 0.471\ X$. Mean regression coefficient of these dogs is significantly different ($0.001 > p < 0.005$) from that of dogs shown in Fig. 1.

FIG. 3. Disappearance of epinephrine from arterial plasma during hour following a constant infusion of 7.12 μg/kg/min. for 60 min. Difference between arterial plasma concentration of epinephrine at each time (C_T) and pre-inj. plasma level (C_0) is plotted logarithmically against time. Calculated regression line is $Y = 1.358 - 0.013\ X$. This regression coefficient is significantly different ($p < 0.0005$) from that of groups in both Fig. 1 and 2.

ume, but less than total extracellular fluid volume. Removal rates indicate that it is not simply distribution into classical fluid compartments, *i.e.*, disappearance from plasma compartment after single injection and from total extracellular fluid compartment after continuous infusion, but other compartments or removal mechanisms are involved.

Plasma disappearance rate following single injection and that within 5 minutes after termination of infusion of epinephrine, as well as maintenance of a nearly constant plasma level when infusion is at the rate of 6 μg/kg/min, demonstrate a rapid rate of catabolism. On the other hand, in the single experiment in which it was followed, the slow rate of disappearance from 5 to 60 minutes after discontinuance of constant infusion suggests slow release of epinephrine from a reservoir, such as might occur following binding to proteins and platelets or entry into cells. Alternative possibilities are that the precipitous fall in blood pressure following cessation of continuous infusion may have been stimulus for release of endogenous catechol amines, thereby prolonging elevation of plasma levels, or may have reduced perfusion of liver and other organs so that destruction of epinephrine was impaired.

Extreme importance of awareness of prompt rate of disappearance of epinephrine is apparent. In animal experimentation or clinical investigation, transiently elevated plasma levels will remain undetected unless appropriately timed samples are analyzed. This has been stressed in diagnosing pheochromocytoma (19).

Summary. Epinephrine (40 μg/kg) injected rapidly, intravenously, disappears from plasma of anesthetized dog in 4 to 6 minutes. Mean half-time for this disappearance rate is 23 seconds. Epinephrine administered by continuous intravenous infusion approaches constant plasma levels in 30 minutes when given at 6 μg/kg/min. Following cessation of a constant infusion, plasma epinephrine concentration falls rapidly for 4 or 5 minutes, then slowly during subsequent hour. Half-time for rapid fall is 38 seconds and for the slow phase, 23 minutes. Inconsequential amounts of epinephrine are excreted in urine.

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Tranquilizing Effect of "Substance P". (24917)

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Because of synergism between "Substance P" (SP) on the one hand and mephenesin(1) (MF) and meprobamate(2) (MB) on the other hand, we have postulated that SP may be a physiological tranquilizer(3). According to their mechanism of action, tranquilizers have been classified by Berger into 2 groups (4): 1) autonomic suppressants which comprise derivatives of phenothiazine, reserpine and diphenylmethane and, 2) central relaxants derived from propanediols such as MF and MB. Our studies indicate that there is a similarity between SP and the central relaxant group of tranquilizers. SP and central relaxants do not possess anticholinergic or antihistaminic action. They counteract strychnine convulsions and prolong hexobarbital sleep. They do not influence conditioned reflexes but relax spastic muscles. The autonomic suppressants on the other hand often produce an opposite effect. Previously we showed that tranquilizing drugs such as chlorpromazine(5), reserpine(6), as well as mephenesin and meprobamate (unpublished), have a marked sedative effect on wild hares kept in a state of fear. Our experiments were designed to find out whether SP was also capable of producing a tranquilizing effect in frightened hares. Wild hares are preeminently suited as experimental animal for evaluation of tranquilizing drugs. When kept in a cage with a dog nearby, the hares develop within one or 2 weeks distinct hyperthyrosis with marked exophthalmus(5,7). The animals make frantic efforts to escape, do not permit being touched or held and move around constantly. They become quiet when no visible observer is present. When exposed to strong light or in presence of an observer their restlessness returns. It has previously been shown that SP had a sedative effect on normal animals(8).

* We thank the Scientific Research Fund of Rectorate of Sarajevo Univ. for providing resources for these studies.

Methods. The hares were divided into 3 groups. Group 1 consisting of 6 control animals were hares hunted down in their natural habitat and sacrificed immediately after capture. Group 2 of 11 hares that were kept in a state of fear for 2 days by the presence of a dog. Group 3 of 7 hares kept in a state of fear for 2 weeks. Group 1 had 20 to 23 units of SP/g of brain. A similar amount was found in brains of hares belonging to Group 3. Hares of Group 2 were given doses of SP, prepared according to Pernow(9) and purified according to Euler's procedure(10). Our preparations of SP contained approximately 3 to 3.5 units/mg. The assay was carried out using a reference standard of SP kindly given to us by Dr. Pernow. SP was given in doses of 100 mg and 300 mg/3 kilos body weight, intravenously.

Results. After receiving SP, all animals became subdued within 5 to 10 minutes. They stopped jumping about and did not react to presence of man or barking of dog. Half an hour later the animals tolerated handling and permitted being held in hand like tame rabbits. Hares given the smaller dose of SP remained tranquilized for 7 hours while those receiving the larger dose remained tranquilized for about 24 hours. Some hares given 100 mg of SP were sacrificed 4 hours later and amount of SP in their brains determined. SP had increased to 30-31 units/g of brain. Similar tests were carried out with another group of hares given meprobamate. The tranquilizing effect of the drug was rapid and intense.

Discussion. It appears that SP has a definite tranquilizing effect. It is important to ascertain the mode of action of SP on the hypothalmus and hypophysis of experimental animals. We hold that SP exerts a depressive action on stress-produced stimulation of the hypothalmus. It is of particular interest that SP in the brain of rats tends to decrease under the influence of both MF and MB. Res-

erpine, on the other hand, a tranquilizer belonging to the group of autonomic suppressants, has no effect on SP of the brain of dogs (11). Our results showed that the amount of SP remains unchanged in hares subjected to fear for 14 days. No attempt was made to investigate the amount of SP in brains after shorter periods of time. That the amount of SP in brains of hares given SP intravenously is increased, indicates that this substance is able to pass the blood brain barrier. Presence of SP in the reticular formation(8,12,13) and especially the high amounts present in substantia nigra (which represents the condensation area of the reticular substance(14)), indicates that SP may represent an active principle closely connected with the function of nerve cells.

The marked tranquilizing effect of SP in hares lends support to the view that this substance belongs to the class of biological regulators of the neuro-endocrine system or that it performs the function of a physiological tranquilizer.

Summary. Wild hares have 20 to 23 units of Substance P/g of brain. This amount does not change when animals are kept 14 days in cages in a state of fear, although hyperthyrosis develops during this time. When Substance P is given intravenously its amount in the brain increases. Wild hares given Substance P intravenously behave like tame rabbits.

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Quantitative Study of Estrogen-Induced Atherosclerosis in Cockerels.* (24918)

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Hyperlipemic atheromatosis has been produced in chickens by subcutaneous implantation of diethylstilbestrol(1). Starting with 3 mos old cockerels, aortic lesions developed in 6 to 7 mos which resembled more closely those occurring spontaneously in birds than the cholesterol-induced type. The 3 types of lesions were fundamentally similar and appeared to differ mainly in concentration of various lipid components(2). Use of 6 wk old birds decreased the possibility that the observed lesions were of spontaneous origin(3). These observations suggested the possibility of developing a quantitative method for study of atherosclerosis. This has been accomplished through time and dose response studies of the atherogenic effect of estrogenic material on young cockerels. Study of the sequence of artery and blood serum changes was made concomitantly.

Material and methods. Approximately 4

* The authors wish to thank Harris D. Webster, Dept. of Pathology, for assistance in evaluating the arteries.

wk old White Leghorn cockerels were used. Each hatch was organized into experimental and control groups. Because of the number required, more than one series of groups is represented in each Table. Therefore, control data are shown in each Table as means of mean values for all control groups with respective standard deviations. A commercial chick growing mash was fed *ad lib.*† Experimental periods were 1 to 56 days. On the first day of a period of 10 days or less, a single dose of estradiol cyclopentylpropionate dissolved in cottonseed oil (ECP)‡ was administered intramuscularly. At the end of experimental period, the birds were rendered unconscious by electrocution and exsanguinated from the right cardiac ventricle. The aorta and other large arteries, including brachiocephalics and iliacs, were dissected to determine grossly the degree of atherosclerosis. Determination of total and free cholesterol, phospholipid phosphorus, total polysaccharides, and alpha and beta lipoproteins was done on pooled serum samples. Degree of atherosclerosis is expressed as 0 to 4 plus for the 7 day or shorter experimental periods. The "% area" of surface of large arteries examined, which showed gross atherosclerosis, was used for periods of longer duration. Total and free cholesterol were determined by our modification of the Sperry and Webb method(4). Phospholipid phosphorus results were obtained by the phosphate procedure of Fiske and SubbaRow(5). The anthrone method of Graff, Greenspan, Lehman, and Holechek(6) with modification of color development procedure was used for determination of total polysaccharides. Alpha and beta lipoprotein determinations were made by paper electrophoresis, using our modification of the method of Langan, Durrum, and Jencks(7). The modified procedure involved use of a larger (.075 ml) serum sample for a higher degree of accuracy, especially in measurement of alpha lipoprotein; the ferric chloride-sulfuric acid cholesterol reagent was prepared fresh daily; paper strips were pre-extracted with mixture of chloroform and methanol; 4 concentrations of cholesterol standard were employed in preparation of a reference curve.

Results. During 56 days of estrogen administration, a small amount of artery involvement occurred with 2.5 mg dose (Table I). Feminizing effects evidenced by relative comb size occurred with .1 mg dose. Extensive plaque formation accompanied by pronounced blood serum changes characteristic of the disease occurred with doses of 5 mg and larger.

The minimum period to produce a high degree of atherosclerosis was investigated next. Although major changes in all measurements were observed at the 5 mg level in the 56-day experiment, dose was increased to 10 mg to insure development of definite atherosclerotic lesions during shorter periods. The effects of 10 mg dose with variable time periods was evaluated. The cockerels used in the groups, representing first and last 7 wks of experiment were from 2 different sources. Therefore, the means of control group-mean values with standard deviations are shown separately for 1 through 7 day, and 8 through 56 day periods (Table II).

Blood serum changes occurred progressively during first 5 days after treatment. However, rate of change was different for individual serum components. This is demonstrated in free to total cholesterol, and cholesterol to phospholipid ratios. In 2 days free cholesterol and phospholipid values had increased so rapidly that the respective ratios reached values which remained almost constant during remainder of time studied. These rapid changes also appear to coincide with appearance of gross atherosclerotic change in the arteries.

All values represent a high degree of change characteristic of atherosclerosis as early as 7 days after administration of ECP, indicating this to be the period of choice for a quantitative method. However, the 10 mg dose appeared too large to provide sufficiently sensitive conditions. A more desirable dose was determined by experiments in which time was constant at 7 days, with dose of ECP as the variable (Table III).

It is of particular interest that atherosclero-

† Little Brothers Chick Growing Mash, Little Brothers, Kalamazoo.

‡ Registered trademark, The Upjohn Co.

TABLE I.* Dose Response of Cockerels to ECP—56 Day Period.

ECP dosage equivalent, mg/bird/10 days (IM)	Atherosclerosis (% area)	Blood serum values: Cholesterol Total (mg %)	Cholesterol % free/total	Phospholipid (mg %)	Cholesterol/Phospholipid	Lipoprotein (mg %) Cholesterol in: Alpha	Lipoprotein (mg %) Cholesterol in: Beta	Total polysaccharides (mg %)
Controls								
.0	.0	106 ± 8	22 ± 1.6	188 ± 23	.57 ± .04	78 ± 7	42 ± 2	74 ± 8
ECP treated								
.0037	.0	99	23	166	.60	79	40	72
.0074	.0	92	21	166	.55	69	37	77
.0370	.0	97	22	179	.54	70	34	72
.1	.0	112	21	221	.51	—	—	82
.5	.0	98	24	213	.46	74	37	75
1.0	.0	86	24	166	.52	73	36	70
2.5	.1	86	23	205	.42	67	46	69
5.0	22.8	383	58	1206	.32	2.4	710	117
7.5	9.2	746	65	1986	.38	—	—	163
10.0	10.3	883	61	2678	.33	1	966	145

* Control group consisted of 60 cockerels; each treated group consisted of 6 or 7 cockerels.

sis occurred 7 days after treatment with ECP at doses which did not cause changes in serum cholesterol, beta lipoproteins, and total polysaccharides. At these lower levels there were small but probably insignificant increases in serum phospholipids. It is also noteworthy that the feminizing effect was observed in all groups.

Attention is called to high degree of atherosclerosis which resulted from 1.25 mg dose. This also is of special interest since the relatively severe artery involvement is accompanied by only minor changes in serum cholesterol, phospholipids, and total polysaccharides. The alpha and beta lipoprotein changes appear to be more extensive.

The quantitative nature of the response to these large and unphysiologic amounts of ECP is further demonstrated by greater blood serum changes associated with severe athero-

TABLE II.† Time Response of Cockerels to ECP.

Exp. period (days)	Atherosclerosis (% area)	Blood serum values: Cholesterol Total (mg %)	Cholesterol % free/total	Phospholipid (mg %)	Cholesterol/Phospholipid	Lipoprotein (mg %) Cholesterol in: Alpha	Lipoprotein (mg %) Cholesterol in: Beta	Total polysaccharides (mg %)
Controls								
1– 7	.00	96 ± 4	19 ± .9	157 ± 9	.62 ± .05	84 ± 2	24 ± 5	64 ± 1.3
8–56	.03	113 ± 9	23 ± 1	200 ± 24	.57 ± .04	86 ± 13	47 ± 7	69 ± 7
ECP treated								
1	.00	148	39	406	.36	22	153	70
2	Suggestive	270	60	962	.28	10	306	94
3	Diffuse	447	67	1711	.26	7	528	121
5	*	662	68	2496	.27	5	728	121
7	*	616	67	2413	.25	3	640	132
8	14.9	711	64	2423	.29	13	1093	142
15	18.6	814	69	2756	.30	0	955	149
22	12.1	932	68	2938	.32	0	1085	186
29	7.9	836	66	2938	.28	4	975	155
43	19.7	632	57	2010	.31	0	725	141
56	10.3	883	61	2678	.33	1	966	145

* Positive—not measurable.

† Control group consisted of 56 cockerels; each experimental group consisted of 6 cockerels. Dose 10 mg at beginning of period. Equivalent dosage each 10 days.

TABLE III.† Dose Response of Cockerels to ECP—7 Day Period.

ECP dosage equivalent, mg/bird (IM)	Atherosclerosis (plus value)	Blood serum values: Cholesterol Total (mg %)	Cholesterol % free/total	Phospholipid (mg %)	Cholesterol/Phospholipid	Lipoprotein (mg %) Cholesterol in: Alpha	Lipoprotein (mg %) Cholesterol in: Beta	Total polysaccharides (mg %)
Controls								
.0	.0	111 ± 6	22 ± 2	167 ± 19	.67 ± .07	89 ± 3	37 ± 7	68 ± 5
ECP treated								
.156	.2	105	25	192	.55	90	38	78
.312	.6	113	23	205	.55	100	30	84
.625	.4	103	22	192	.54	81	47	75
1.250	2.2	127	24	229	.55	50	112	84
2.5	2.8	216	30	390	.55	17	233	92
5.0	2.4	423	58	1095	.39	—	—	113
7.5	2.0	578	66	1560	.37	2	664	135
10.0	*	616	67	2413	.26	3	640	132

* Positive—not measurable.
† Control group consisted of 39 cockerels; each experimental group consisted of 5 or 6 cockerels.

sclerosis at 2.5 mg level. Maximum artery involvement appears to have been reached at this dose level; however, additional increases in amount of ECP resulted in increasingly greater serum changes.

These results (Tables I and III) also suggest that atheroma, present 7 days after initial treatment, regressed as treatment was continued through 56 days Even the lowest dosage level (.156 mg) resulted in atherosclerotic change 7 days after treatment (Table III), while no atherosclerosis was observed at doses of 1 mg or less after 56 days (Table I).

It is of major interest that intramuscular administration of 2.5 mg of ECP to approximately 4 wk old cockerels at beginning of 7 day period are experimental conditions that constitute a practical quantitative method for study of atherosclerosis.

Summary. 1) Results of dose and time response studies in quantitation of atherogenic effect of pharmacologic amounts of estrogen on young cockerels have been used in formulating a method for the study of atherosclerosis. 2) The method is based upon response of immature birds to 2.5 mg of estradiol cyclopentylpropionate dissolved in cottonseed oil (ECP) administered intramuscularly at beginning of 7 day experiment. 3) Results suggest that feminizing effect and some degree of atherosclerosis result from ECP dosage levels lower than those required to produce hypercholesterolemia and other blood serum changes associated with atherosclerosis. There are indications that atheroma may regress with time.

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Role of Rat Liver in Nonshivering Thermogenesis.* (24919)

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Hart(1) has recently reviewed evidence indicating that, when exposed to cold, cold-adapted rats increase their heat production without shivering. The sympathetic nervous system and adrenal medullary hormone are important in mediation of this thermogenesis (2), but the origin of increased heat production remains unidentified. Visceral tissues as a whole seem to have been excluded as important sources of chemical heat production(1); however, elevated oxygen consumption(3-7) and increased succinoxidase activity(3), as well as higher temperature(8,9), of the liver in cold-adapted rats have been reported. Our experiments were designed to determine whether liver is the primary organ in chemical heat production or whether metabolic activity of liver is secondary and supportive to increased metabolism of other tissues.

Methods. Oxygen consumption, rectal and liver temperatures, relative oxygen tension of the liver, and liver blood flow were measured in 26 male Sprague-Dawley rats weighing 318-437 g. Sixteen animals were cold-adapted (10). Experimental procedures were the same as used by Cottle and Carlson(10) with addition of measurements of oxygen tension and liver blood flow. Production of heat through muscular activity was prevented by curarization. A thermocouple of No. 30 copper-constantan wire was placed between liver lobules to measure temperature of the liver. Oxygen tension was observed by a platinum electrode inserted into liver tissue; a microammeter and a calomel half cell were included in the circuit. The technic was similar to that described by Montgomery(11). Liver blood flow was determined from clearance curve of radioactive chromic phosphate injected into the external jugular vein(12). Chromic phosphate suspended in normal saline solution was injected in amounts of .1-.3 ml, determined on basis of radioactivity. Blood was obtained by snipping off the tail. Samples (0.0078-0.0130 ml) were taken every minute for first 10 minutes after injection and every 5 minutes for following 30 minutes. Blood was transferred to gilt metal dishes, 2.5 cm in diameter, and mixed well with tap water, poured into the dishes beforehand. After complete drying of blood, radioactivity was determined with a Nuclear Instrument DS5-7 scintillation probe. Measurements were corrected for background radioactivity, and calculated in terms of activity/0.013 ml of blood. This activity was plotted as logarithm against time. The slow components were subtracted, and the half time for the fast component was taken directly from the graph. This disappearance constant (k) was calculated from the half time ($T_{½}$) by the formula, $k = 0.693/T_{½}$ (12). Minimum values for liver blood flow were calculated from the disappearance constant and the blood flow. The results were expressed as blood flow/unit volume of tissue/minute. Blood volume was estimated to be 55.6 ml/g body weight(13). Two series of experiments were performed. In the control series, the room temperature was about 30°C, and radioactive chromic phosphate was injected 60 minutes after test began. Five warm-adapted and 7 cold-adapted rats were used. In the experimental series, room temperature was kept at about 30°C for the initial 60 minutes of test, then lowered to 5°C for 70 minutes and held at that temperature. The active chromic phosphate was injected 30 minutes after cooling was started. Five warm-adapted and 9 cold-adapted rats were used.

Results. Liver blood flow. In the uncooled series, liver blood flow in warm-adapted and cold-adapted rats did not differ significantly (Table I). In the cooled series, however, liver blood flow of the cold-adapted rats was significantly greater (nearly at 1% level) than that of warm-adapted ones. In other words,

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TABLE I. Values of Liver Blood Flow Related to Oxygen Consumption and Rectal Temperature.

Rat No.	Liver blood flow (cc/g/min.)	O_2 consumption (cc)	△T (rectum), °C	Liver blood flow (cc/g/min.)	O_2 consumption (cc)	△T (rectum), °C
			Series tested at 30°C			
	Warm-adapted			Cold-adapted		
1	.58	6.3	−1.9	.64	13.0	− .6
2	.65	5.0	−1.1	.71	10.7	− .8
3	.72	5.5	−2.5	.95	11.7	− .4
4	.84	6.3	−2.5	.97	8.5	− .2
5	.99	7.7	− .6	1.04	11.1	− .6
6				1.05	11.5	− .5
7				1.06	11.3	−1.3
8						
9						
Mean	.76 ± .14	6.1	−1.7	.92 ± .15	11.1	− .6
			Cooled series			
	Warm-adapted			Cold-adapted		
1	.45	5.9	−8.8	.65	16.5	−5.0
2	.58	6.0	−7.1	.67	15.2	−1.9
3	.62	5.3	−7.1	.78	19.5	−3.6
4	.64	7.1	−9.7	.86	15.0	− .4
5	.66	6.3	−8.2	.89	13.5	−1.7
6				.98	15.6	−1.5
7				1.04	19.0	−2.1
8				1.24	15.0	−2.1
9				1.90	15.5	−3.4
Mean	.59 ± .08	6.1	−8.2	1.01 ± .38	16.1	−2.4

△T = Difference in temperature readings at 1 hr and end of 2 hr test.

greater liver blood flow in cold-adapted rats was observed only when rats were in a cold environment. A representative example from each cooled group is shown in Fig. 1 and 2.

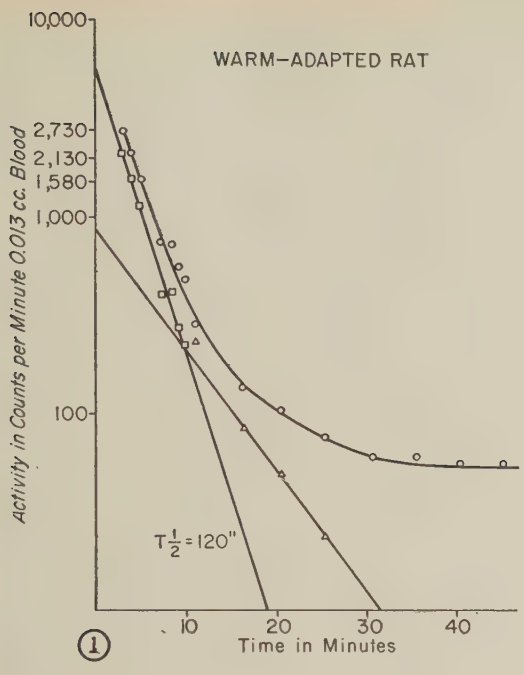
Oxygen consumption. Both warm-adapted and cold-adapted rats in the uncooled series showed a slightly lower oxygen consumption during the latter 60 minutes of test than during initial 60 minutes. In the cooled series, the warm-adapted rats fail to increase their oxygen consumption as cold-adapted animals did, as previously reported(10).

No significant differences between liver and rectal temperatures were observed in any one test. However, both liver and rectal temperatures decreased towards the end of the test, and this lowering was more pronounced in warm-adapted rats, especially those which had been cooled.

Oxygen tension in liver. No significant differences in O_2 electrode current readings either between warm-adapted and cold-adapted rats or between individual animals were seen. However, the value could be decreased by asphyxia and increased by inspiration of pure O_2.

Discussion. While there is no question that capability for heat production is enhanced in cold-adapted rats, the site of thermogenesis cannot be partitioned between various tissues. The greater liver blood flow in cold-adapted rats when they were exposed to cold, is undoubtedly linked to their greater metabolism, but the mechanism of the linkage cannot be determined from these experiments. That liver blood flow in cold-adapted animals increased approximately 10% while metabolism increased approximately 50% would indicate that if the liver were supplying the heat it would require a large oxygen extraction from blood and an increase in temperature. As previously indicated(14), liver metabolism would have to increase 6-fold to provide this heat. It seems impossible that the liver produces all of the additional heat.

Previous experiments(2) showed metabolic response to cold in the cold-adapted rat to be mediated *via* the sympathetic nervous system and indeed the metabolic response to noradrenaline changes markedly(15). There is no elevation of blood sugar or increase in blood lactic acid during thermogenesis in re-



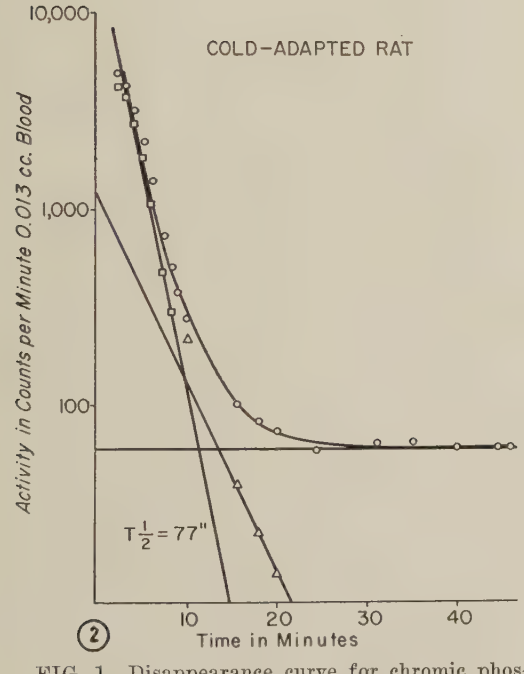


FIG. 1. Disappearance curve for chromic phosphate inj. intrav. ○ = actual experimental data; △ = difference between experimental data and slowest component, and □ = difference between experimental data and 2 slow components.

FIG. 2. Disappearance curve of chromic phosphate inj. intrav. ○, △, and □ have same meaning as in Fig. 1.

sponse to cold(15). If the response described by Cottle and Carlson(10) results from muscle metabolism, as suggested by Depocas(16), the mediation *via* sympathetic nervous system is an unexplored, though attractive, mechanism. Adrenaline will increase muscle metabolism, but this increase is accompanied by production of lactic acid(17). Freund and Jansen directed attention to the role of the sympathetics in chemical thermogenesis in muscle, although the manner of mediation was not specified(18). Donhoffer *et al.*(19) observed that the metabolism of 7 narcotized rats cooling at approximately 20°C fluctuated with a periodicity of 15-20 minutes. The authors show that the liver and intestine temperatures follow this fluctuation while muscles continue to cool.

Summary. Comparative studies on liver blood flow, oxygen consumption, liver and rectal temperatures, oxygen of liver were conducted on cold-adapted and warm-adapted rats. Muscular activity of all rats was blocked by curare. Liver blood flow was measured by blood clearance curve of radioactive chromic phosphate injected intravenously No difference in temperature between rectum and liver was found in a given rat in both groups. Liver blood flow in cold-adapted rats did not differ from that of warm-adapted ones so long as tests were performed in a warm room (30°C). However, liver blood flow in cold-adapted rats was considerably greater than that of warm-adapted ones when animals were cooled, though not sufficient to account for the excess oxygen consumption.

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Renal Excretion of Calcium and Phosphate in the Mouse as Influenced by Parathyroids.* (24920)

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Over recent years, influence of the hormone of parathyroids on bone metabolism has been reemphasized. McLean(1) described parathyroid effect on bone as a "feedback" mechanism for maintaining serum calcium levels. Our reports(2,3) have also emphasized importance of the action of parathyroids on bone. However, for complete picture of parathyroid action, influence of the hormone on kidney function cannot be disregarded. Greenwald's classic observation(4) that parathyroids directly influence renal phosphate excretion has been amply confirmed by many workers, and formed the basis for widely held theory of parathyroid function(5). That parathyroids might also exert a direct influence on renal calcium excretion has been for the most part ignored. Nevertheless Jahan and Pitts(6) reported increased tubular reabsorption of calcium following parathyroid administration in dogs; Talbot *et al.*(7) suggested that parathyroid extract raised renal calcium excretion thresholds. Talmage *et al.* (8,9) studying influence of parathyroidectomy on parathyroid extract administration on calcium excretion interpreted their data as changes in renal threshold for calcium, which caused altered excretion rates until plasma levels equilibrated with new thresholds established by changes in circulating parathyroid hormone concentrations. The work here presented was undertaken to study this effect in yet another species with hope of further emphasizing the overall role of parathyroids on renal excretion.

Methods and materials. Approximately 240 Rockland mice weighing 20-25 g were used. In most experiments, mice were placed on calcium and phosphate free diet with 14 mg Ca/100 ml in drinking water for 2 to 3 days prior to use. In a few parathyroid extract experiments, animals were maintained on stock diet. Parathyroids were removed individually under Nembutal anesthesia, using jewelers' forceps and dissecting microscope. A midline incision was made just posterior to larynx and thyroids exposed. The single pair of parathyroids lie on lateral border of thyroids, usually midway of length of the gland. Occasionally glands are slightly dorsal to thyroids. Parathyroid extract when given was injected subcutaneously in 6 doses at 1 hour intervals. Radioisotopes were injected intraperitoneally. In a typical experiment, animals were given radioisotope and 1 ml water intraperitoneally at beginning of experiment. Mice, in groups of 4 to 6, were placed on metabolism funnel and urine collected for 2 hour periods. Additional doses of 1 ml water were given at start of each collection period. Parathyroidectomy or parathyroid extract administration was performed as indicated in re-

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TABLE I. Parathyroid Influences on Renal Excretion of Ca^{45} and Phosphate in Mouse.

	No. of animals (groups)	Periods I	II	III	IV	V	VI	VII
Ca^{45}, total radioactivity/hr/mouse	PTH 65 (17)	868 ± 153	520 ± 82	485 ± 71	523 ± 100	308 ± 63	475 ± 115	382 ± 156
	CON 71 (17)	1000 ± 151	1000 ± 159	1000 ± 110	1000 ± 90	1000 ± 115	1000 ± 91	1000 ± 143
	PTX 33 (8)	3158 ± 1800	4302 ± 1774	7018 ± 1480	3175 ± 952	2458 ± 240	1341 ± 598	902 (1351-451)
Ca, mg/hr/mouse	PTH 65 (17)	.0071 ±.0010	.0053 ±.0006	.0057 ±.0005	.0033 ±.0005	.0033 ±.0005	.0031 ±.0004	.0047 ±.0004
	CON 71 (17)	.0094 ±.0013	.0056 ±.0006	.0055 ±.0009	.0067 ±.0010	.0050 ±.0008	.0055 ±.0008	.0043 ±.0008
	PTX 33 (8)	.0080 ±.0016	.0068 ±.0010	.0097 ±.0016	.0137 ±.0026	.0153 ±.0014	.0086 ±.0016	.0050 ±.0005
PO^4, mg/hr/mouse	PTH 65 (17)	.14 ±.03	.18 ±.04	.21 ±.02	.21 ±.02	.22 ±.03	.19 ±.02	.19 ±.04
	CON 71 (17)	.12 ±.02	.09 ±.02	.11 ±.01	.14 ±.02	.12 ±.02	.17 ±.02	.17 ±.02
	PTX 33 (8)	.04 ±.01	.03 ±.01	.03 ±.01	.04 ±.01	.04 ±.02	.07 ±.04	.07 (13-.01)

Parathyroid extract (PTH) administered in 6 hourly doses beginning with 3rd hour.
Parathyroidectomy (PTX) performed prior to first collection period.

Values given with stand. error $= \pm\sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

Collection each 2 hr.

sults. Calcium determinations were made with flame spectrophotometer by procedure involving precipitation with ammonium oxalate and redissolution with perchloric acid. Phosphate determinations were made by the method described by Allport and Keyser(10). Ca^{45} determinations were made on dried aliquots of urine, using a flow counter. A small amount of sucrose solution was added to samples before drying to bring total sample weights into a range where sample variation in self-absorption was less than overall experimental error. Sr^{85} determinations were made with NaI scintillation counter and pulse height analyser.

Results. Phosphate excretion changes. Expected changes in phosphate excretion, noted for other mammalian species following parathyroid extract administration or parathyroidectomy, were seen also in the mouse. Shortly after parathyroidectomy renal excretion values dropped to approximately one-third of control values, while administration of 60 to 90 I.U. of parathyroid extract doubled phosphate excretion rates (Table I). Length of experimental period was 12-14 hours. By end of this period excretion values in both experimental groups appeared to return toward normal values. This can be noted in the large standard error seen in parathyroidectomized groups after 10th hour. The magnitude of this error was due to some groups showing a complete return to normal excretion values.

Calcium excretion changes. Changes in calcium excretion rates in the experimental groups were in the opposite direction to phosphate changes. These changes agree with data previously reported for the rat(9,10). The increase in calcium excretion following parathyroidectomy is interpreted as due to decreased tubular reabsorption for calcium ions causing a temporary increase in urinary calcium values. For groups receiving parathyroid extract, the reverse was true, calcium excretion dropping temporarily (Table I). By end of experimental period all calcium excretion values were within control range.

To indicate further these calcium changes, radiocalcium (Ca^{45}) was administered at be-

ginning of experimental period. Though values for radiocalcium excretion were not directly proportional to total calcium excretion throughout experimental period, radioactivity changes paralleled those for total calcium (Table I). Since injected radioactivity was far from equilibrium with calcium in the body, radiocalcium changes can only be used as a further indication of direction of change in renal calcium excretion and not for degree of change. A few experiments were run using Sr^{85} as indicator of calcium changes. Changes in radiostrontium excretion followed the same pattern as for radiocalcium. The reason for use of this isotope has been previously explained(2).

Discussion. Indications that the hormone of parathyroids directly influences renal calcium excretion have now been given for man (7), dog(6), rat(9), and in our studies for the mouse. Results in regard to renal calcium effects are never as spectacular or "clear-cut" as are effects on phosphate excretion. Reasons for this are 2-fold. Calcium excretion values are in the order of magnitude of 10% of that for phosphate. Calcium excretion rates are, therefore, normally very low, and because of excretion variability, changes are difficult to establish. The second reason concerns the relationship of handling of these 2 ions by kidney and bone. With higher hormone titers, increased phosphate release from bone is coupled with decreased renal phosphate reabsorption. Thus excretion changes are exaggerated. Conversely, an outpouring of calcium from bone is coupled with presumably simultaneous increased tubular reabsorption and therefore excretion values may change little or not at all despite marked changes in handling of this ion by the kidney. In dogs actual excretion rate changes for calcium under varying parathyroid states have not as yet been shown though increased tubular reabsorption following parathyroid administration has been claimed(6). In both rat and mouse temporary changes in renal excretion rates can be demonstrated in both hypo- and hyperparathyroid states but are seen only until bone-plasma equilibrium adjusts to the new tubular reabsorption rate in the kidney.

If the generally assumed function of parathyroid hormone, namely maintenance of normal plasma calcium values, is correct, this certainly would be done most efficiently by the hormone if, with removal of calcium from bone, a simultaneous increase in renal tubular reabsorption occurred to reduce loss through the kidney. The renal phosphate effect, however, being in the opposite direction, not only permits loss *via* the kidney of phosphate removed from bone with the calcium, but also (by excretion of additional phosphate) prevents blood from reaching saturation point for these 2 ions.

Summary. Changes in renal excretion rates of calcium and phosphate, as affected by parathyroidectomy and parathyroid extract administration, were studied in mice. Parathyroidectomy temporarily increased urinary calcium values to 3 times control values, while decreasing phosphate excretion to ⅓ normal values. Urinary excretion of both ions returned to within normal range by 14 hours after start of experiment. Parathyroid extract administration produced opposite changes of about the same magnitude. Urinary calcium values decreased while those for phosphate rose. These changes were also temporary. Results are interpreted as due to changes in renal tubular reabsorption rates for the 2 ions, causing altered excretion rates until plasma levels adjust to the new renal thresholds. Particular emphasis is placed on changes in renal calcium excretion. These studies demonstrate parathyroid influences on handling of calcium by the kidney for a fourth mammalian species, suggesting the importance of this little considered aspect of the action of parathyroid hormone in overall parathyroid physiology.

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Effects of Extremely High X-Ray Intensities and Dosages on Mice. (24921)

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Considerable information has been collected on effects of low and medium high dosages of ionizing radiation upon living organisms. Recently, interest has developed in delivering large amounts of such radiant energy to animals, due in part to ability to produce sufficiently high energy flux, and to increasing recognition that interactions between living organisms and ionizing radiation can now be investigated which might not be observable at lower radiation dosages and dose rates(1-6). However, data obtained with extremely high dosages and/or high intensity x-rays is rather scarce(7). Extremely high x-ray dosages and intensities referred to in this paper connote dosages of 10^5 r or more, and intensities of 10^5 r/minute or greater.

Methods. The x-ray tube was placed inside a lead lined cabinet with lead glass window. Temperature in cabinet stayed reasonably constant over considerable time. A strong fan served to remove ozone and stabilize temperature. High intensity radiation was produced by Machlett AEG 50 Beryllium window tube at 45 KVp and 40 mA. No filtration was added. Distance from target to animal's skin was 3.6 cm with dose rate of 2.1 x 10^5 r/minute. X-ray output of tube was repeatedly checked with chemical dosimeter (8). For this purpose ferrous sulfate reagent (0.5 ml, 5 mN) was placed in small glass cups fitting snugly into lucite holder and irradiated for various time intervals† (distance from target to level of liquid = 3.6 cm). Depth of fluid was 2-3 mm. Following irradiation, 0.3 ml of ferrous sulfate reagent was removed and diluted with 3 ml of 0.8 N sulfuric acid. Optical density after irradiation was compared to that of non-irradiated samples of solution in Beckman spectrophotometer at wave length setting of 3020 Å. The dose of 2.1 x 10^5 r/min thus obtained is representative of energy absorbed in first few mm of skin over area of 2.25 cm^2. This procedure compares quite well with mesh ionization chamber measurements performed earlier at same x-ray energies and similar exposure dose rates. X-ray absorption measurements were carried out with same type of dosimeter by placing shallow containers of liquid inside a lucite holder and covering holder with pieces of mylar or lucite varying in thickness 12 μ to 2.45 cm. In this particular instance, distance from tube window to liquid level was 5.5 cm and remained constant throughout. The resulting absorption curve (Fig. 1) shows that approximately 85% of x-rays are absorbed in the first cm of tissue. Output constancy measurements with a CdS crystal dosimeter were $\pm$ 5%; however, after tube had been in operation for about 150 to 200 hours a gradual reduction in output was observed to as low as 1.3 to 1.1 x 10^5 r/min. Some of our repeat

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† Amount of x-rays delivered to ferrous sulfate solution did not exceed 5 x 10^4 r and therefore should be within useful range of this dosimetric method. This was confirmed by 2 measurements performed with Ceric-Cerous dosimetry system.

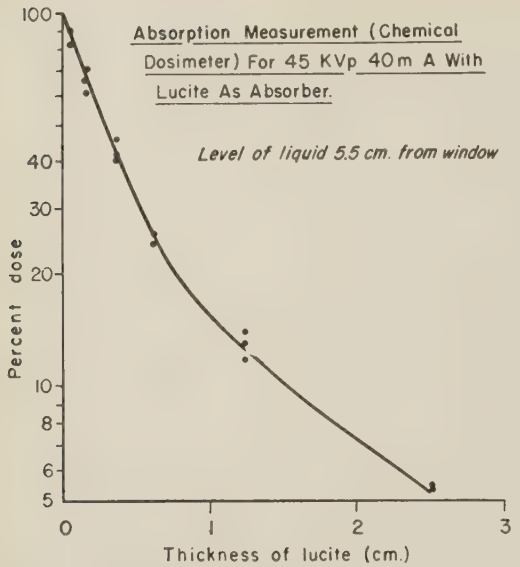


FIG. 1. Absorption of 45KV unfiltered x-rays. (Be-window only) in Lucite.

experiments were made during this period of decrease and are marked accordingly. Other serious dose variations may occur due to slight changes in location of animal from proper target distance (3.6 cm TSD for all mice) due to inverse square effects. Despite proper marking devices, this can easily happen due to changes in breathing, convulsions, and struggling motions of animals during exposure. Furthermore, dose distribution over area covered by x-ray beam at the particular target distance is not uniform (Table I). Thus determination of absorbed energy is difficult and only approximate values can be obtained. Temperature measurements were carried out with a Thermister Tele-Thermometer (Yellow Springs Instrument Co.). The hypodermic needle type thermistor probe was always inserted into the thigh of hind leg of mouse, which was protected by quarter inch lead shield. This shield extended beyond the tip of the thermistor probe but did not touch the skin. This location of the probe was used in either abdominal or head irradiation. In some later experiments rectal temperature readings were also recorded. A total of 130 mature CFW mice have been studied, averaging about 25 g with extremes from 19-32 g. Food and water were not restricted until just before each experiment. Animals were secured, back down, onto small boards by adhesive tape over individual limbs. Mice were exposed to radiation of either the head or lower parts of body subsequently called abdominal irradiation (Fig. 1). The head of a 25 g mouse approximates about 1 to 1½ cm as measured from the mandibular bone to up-

TABLE I. Dose Distribution.

Distance from axis of beam, cm	% dose parallel to tube axis	% dose right angle to tube axis
2.5		.3
2.0	.4	2.3
1.5	.6	18.
1.0	30.	36.
.5	80.	80.
.0	100.	100.
.5	80.	80.
1.0	50.	40.
1.5	27.	25.
2.0	16.	2.7
2.5	1.2	.3
3.0	.3	

Field size 4 cm diameter.
CdS detector target distance = 3.8 cm.

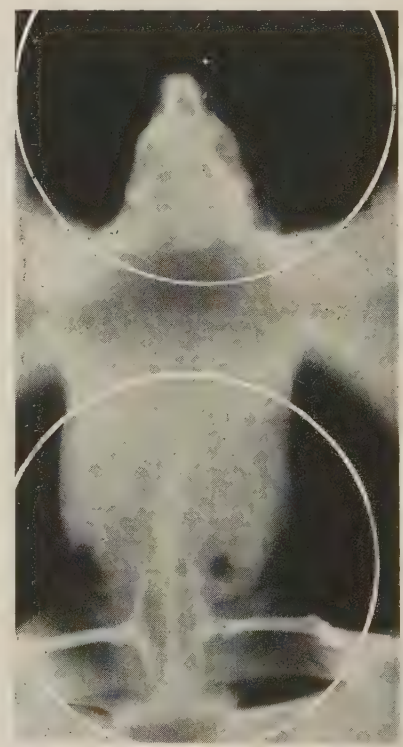


FIG. 2. Radiograph taken with 25 kv x-rays shows areas of mouse body exposed to x-rays. In this figure head and abdominal treatment fields are shown on one animal.

per parietal bone of skull. Body depth through the abdomen to dorsal side is somewhat greater. Mice that were difficult to handle were injected with small amounts of Nembutal. Additional radiation experiments revealed no variation in survival time of these from that of non-anesthetized animals. To obtain reliable temperature measurements, all animals were placed under the tube for 3 to 10 minutes prior to x-ray treatment. Normally, little change in temperature was observed. The temperature of target cooling water was regulated to vary within 1 degree (C°) of irradiation cabinet temperature, before and during operation of tube. This cooling water also kept entire headpiece of x-ray tube at constant temperature by circulating the cooling water through water jacket surrounding tube head, with exception of the window. The following studies were carried out during and after exposure of mice: A. Determination of survival time following: 1. Head exposure only at normal body temperature. 2. Abdominal exposure at normal body temperature. 3 and 4. Repetition of 1 and 2, but at artificially lowered body temperature. B. Recording changes of body temperature.

Results. Mean survival time of mice after a surface‡ dose of 2 x 10^6 r (2.1 x 10^5 r/min) to head or abdomen was determined. Mean survival time of head irradiated animals was 11.8 ± 1.4 minutes at normal body temperature. A survival time of 14.9 ± 2 minutes followed irradiation of abdomen. This somewhat prolonged survival in contrast to head irradiation was rather persistent throughout. Above values demonstrate mean survival times without regard to body volume bathed by x-ray beam. If we examine survival times in respect to total absorbed energy, assuming that the abdominal area exposed to radiation is about 3.6 times as large(9) as that of head, much greater sensitivity of brain to irradiation under these circumstances becomes clearly evident (Table II). Equal amounts of absorbed energy by either of the 2 body portions results in much shorter survival time for head irradiation than for abdominal treatment. This greater response of brain has also been observed with a dose of 10^5 r by Rajewsky(7), who recorded a survival time of approximately 9 hours following irradiation of mouse rump in contrast to 90 minutes after head irradiation.

TABLE II. Mean Survival Times of Mice. 2 × 10^6 r (2.1 × 10^5 r/min.)

Irradiated body parts	Temp. of body	Wt of body part(9), g	Mean survival time (min.)
Head	Normal	4- 6	11.8 ± 1.4*
Abdomen	"	15-16	14.9 ± 2.0
Head	Lowered	4- 6	104.0 ± 17.2
Abdomen	"	15-16	116.0 ± 17.0

* Stand. error of mean.

We were also concerned with the toxic amounts of ozone produced by highly intense x-ray beam. To eliminate any possibility of accelerated death from this factor, survival times of animals exposed to the beam without any ventilation in the x-ray tube cabinet, were compared with mice irradiated under identical conditions, but with constant use of forced air stream between tube window and head (in addition to cabinet ventilation). The data so obtained exhibit no discrepancy in survival time between the 2 groups; this is in accord with findings of Brace and Andrews(5). However, one can be reasonably certain that the level of ozone produced by such highly intense ionizing radiation would have a profound effect on animals if they lived longer(10). In our experiments, radiation damage was so severe as to overshadow any other effects.

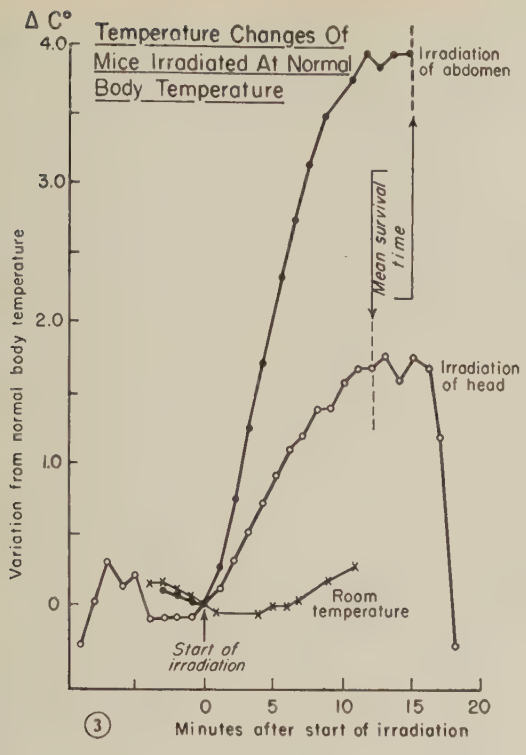
Body temperature exhibits considerable change during either type of (head or abdomen) irradiation; therefore, in additional experiments mouse body temperature was reduced 4-8°C during exposure. This was achieved by placing blocks of ice around the animal, whose entire back soon was bathed in ice water. This caused no interference with x-ray beam since the mice were tied down with backs resting on restricting board. Survival time obtained in this manner was much prolonged, 7-8 times for head as well as for abdominal irradiation (Table II). Whether this phenomenon is in direct relationship with keeping body temperature at levels more com-

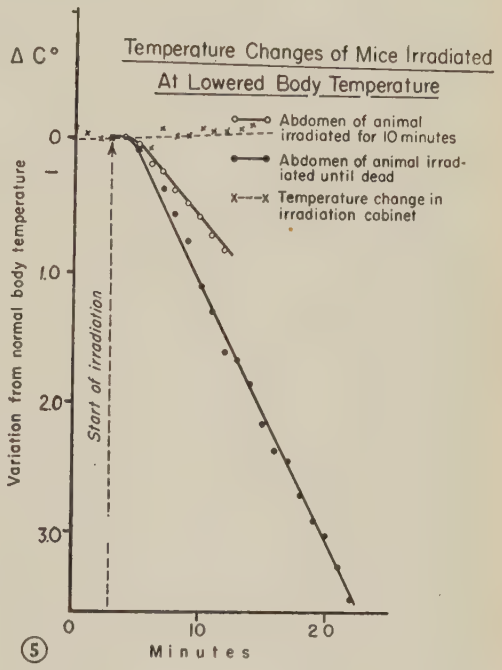
‡ Surface dose in this case means dose integrated over first 2-3 mm depth of ferrous sulfate solution.

patible for animal organism, or whether some indirect kind of protection for body chemistry is afforded by cooling, remains to be answered. Mice irradiated at lowered body temperature provided opportunity to study over a longer period of time the effects of radiation, whereas the others had usually expired by time of removal from the animal holder. Mice (cooled) abdominally irradiated showed no extraordinary symptoms that had not been observed in animals exposed to smaller dosages of radiation. The animals were hunched, huddling together, as if seeking warmth. Periods of lethargy intermingled with short intervals of great excitability, the latter becoming less and less frequent. Water and food were consistently ignored. Respiration became increasingly difficult toward the end, but was continued often over long periods of time (1-3 hours) though the animal was on its side or back, and apparently unconscious. In some instances, the hind extremities appeared to be paralyzed, but this could not be confirmed. Pain perception in the tail was maintained. Head irradiated (cooled animals) demonstrated identical symptoms to those discussed above. However, paralysis of one or other of the extremities occurred more often. Periods of excitement were more frequent and longer, and the animals demonstrated complete loss of orientation. Circular, screw or eel type convulsive motions at surprising speeds were noted in most instances(11). The mice maintained only very labile balance when able to stand at rest, or remained as placed when not in a stage of excitement. Many of these sequelae seem to be the same or similar to those observed by Gerstner *et al.*(12) at lower x-ray dosages (12,500 r). Cooling protracts the above mentioned disturbances and symptoms in head irradiated animals. This became evident in 2 instances when such mice were freed from the animal holder before death occurred and similar screw type motions were observed.

Changes in body temperature. One can observe a reduction of body temperature as a function of dose and time after exposure to medium and high dosages of x-rays, Langham *et al.*(2). The interest in these particular experiments was to determine variations of temperature during irradiation. The average increase of body temperature after 10 minutes of head irradiation (2×10^6 r) was 1.7°C whereas it amounted to 3.9°C for the same irradiation time of the abdominal area (Fig. 3). This rise in temperature could perhaps in part be attributed to the absorbed energy of the high intensity beam and heat from the tube window. To compare these temperature changes with those from other heat producing sources, the following experiments were carried out. Temperature increase of the x-ray tube window during operation was determined with a copper-constantan thermocouple. Then a metal disc of equal diameter as the tube window was heated to about the same temperature (measured with the same thermocouple) as the window. This was achieved by a 15 watt electric bulb housed in a plastic container to which the disc was attached. The abdomen of the mouse was then placed first under the lamp heated disc (1.6 cm from disc) and temperature changes recorded. After temperature returned to normal the same mouse was moved under the x-ray tube and irradiated about the same amount of time at 1.6 cm from the window (3.6 cm from target) and again temperature measurements were made.

At the time these particular experiments were performed, we had to take into consideration that the tube output was reduced to about 1.2×10^5 r/min. The results obtained indicate a greater body temperature increase with x-irradiation than with the simulated treatment using the lamp: however, temperature increase is smaller than observed previously as one would expect with reduction of x-ray tube output (Fig. 4). These observations could be interpreted to mean that part of the temperature rise in the animal is due to heat from the window, whereas the remaining part must be due to the absorbed radiant energy (ionizing) and/or the reaction of some biological process in the animal. This latter assumption seems to be supported not only by the fact that body temperature drops considerably despite continuation of treatment after the animal has expired, but also by phantom measurements. Such a phantom consisted of a mouse shaped thin plastic bag filled with 10 or 20 ml of water. This bag was irradiated





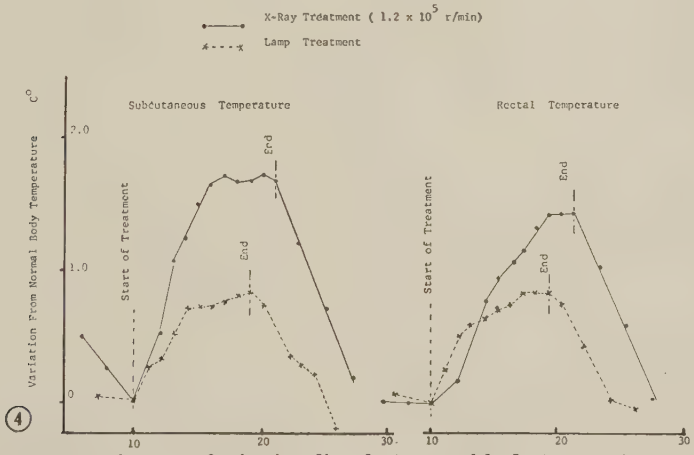


FIG. 3. Temperature changes of mice irradiated at normal body temperature.

FIG. 4. Variation of body temperature of mice exposed to x-ray treatment or heat treatment. (Abscissa: time in min.)

FIG. 5. Two examples of temperature changes of mice irradiated at subnormal body temperature.

with x-rays or the lamp in the same manner as the mice discussed earlier. The temperature changes recorded under these conditions were about equal for both sources. However, one of the problems has not been solved, namely determination of the true x-ray window temperature. Though both thermocouple measurements (x-ray window and metal disc on lamp) were adjusted to show equal temperature readings, it was impossible with this arrangement to find out how much of the observed temperature increase is caused by the

heat of the x-ray window alone and to what extent absorbed x-ray energy in the thermocouple itself contributes to the temperature change. It could be very well possible that the actual window temperature is lower than the thermocouple indicates.

Fig. 5 shows changes of body temperature during x-ray exposure of animals cooled by ice water. In all instances temperature was reduced below normal, and life was prolonged regardless of treatment area. At the very least a doubling of dose was required to kill the animal within 25 minutes.

Additional information has been sought by investigating changes in breathing pattern, electrocardiogram tracings, as well as blood changes and histological picture, since the above findings present a variety of possible reasons for super-acute radiation death of the animals. Results obtained in these studies are being analyzed.

Summary. The effects of extremely high x-ray dose rates and dosages on mice irradiated at normal and subnormal body temperatures were studied. Considerable lengthening of survival time is obtained when body temperature is lowered during irradiation. A slight elevation of body temperature during irradiation was noticed.

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Carbohydrates and Gastrointestinal Absorption of Radiostrontium and Radiocalcium in the Rat.* (24922)

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Ability of lactose to increase gastrointestinal absorption and retention of calcium has been known for many years and has been adequately confirmed under various conditions and in different species. The effects of lactose on calcium metabolism and other physiological functions have been reviewed recently by Duncan(1) and Atkinson *et al.*(2). Although the action of lactose has been widely studied, little attention, with one exception, has been given to the influence of other sugars and sugar derivatives on calcium. Fournier and his colleagues(3-5) determined the net retention of calcium in rats as influenced by ingestion of sugars by use of conventional calcium balance technic. Certain sugars, in addition to lactose, were capable of enhancing calcium retention in the growing and lactating rat; *e.g.*, xylose, mannose, raffinose, mannitol and sorbitol. It was not feasible with calcium balance method to determine extent

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to which carbohydrates increased gastrointestinal absorption or acted by direct effect on skeletal growth. The work presented here, based on tracer procedures, distinguishes between skeletal and absorptive effects. The technic was used to estimate effect of various sugars and sugar derivatives on gastrointestinal absorption of radiocalcium and radiostrontium in a way similar to that previously described for study of the influence of various amino acids on intestinal absorption(6).

Materials and methods. Male rats of Wistar strain weighing about 90-110 g were fasted 24 hours with only drinking water available. At this time, the rats were lightly anaesthetized with ether and given, by gavage, 1 ml of test solution containing 20 μc Ca^{45}, 2 μc Sr^{85}, 0.42 millimole of test substance and 0.04 millimole of calcium chloride. The animals were killed at 24 hours after dosing and the femurs removed for radioassay. Calcium 45 was determined by oxalate-precipitate method described by Comar(7) and Sr^{85} was measured by counting excised whole femur in a well-type scintillation detector. It has previously been shown that the appearance of Ca^{45} and Sr^{85} in bone 24 hours after oral administration is a measure of gastrointestinal absorption(6) and, more specifically, that lactose administered under similar conditions does not directly influence movement of alkaline earths from blood to bone(8). In the latter study, the ratio of femur Sr^{85} to absorbed Sr^{85} as directly measured was not affected by presence of lactose. Radioassay data are expressed in terms of "% of administered dose" or as "% of ingested dose."

To study further this lack of lactose effect on skeletal metabolism, a balance-type technic was employed in which young growing male rats on Rockland stock diet were placed on diets that contained either 30% sucrose or 30% lactose. When rats were placed on experimental diets, the drinking water was replaced with distilled water containing 0.02 μc Sr^{85}/liter; the rats were continued on this regime for 7 days. By use of graduated drinking tubes, the volume and therefore the amount of activity ingested was determined. Radiostrontium or radiocalcium ingested in this manner has been shown to equilibrate with, and be metabolized similarly to, dietary calcium in the young growing rat(9). The feces and urine were collected separately over the 7-day period and at this time the rats were killed and the contents of the digestive tract was removed and combined with the pooled feces. The pooled feces plus digestive tract contents, pooled urine samples and the left femur from each rat were radioassayed for Sr^{85}.

Carbohydrates were obtained from Nutritional Biochemicals Corp., and used without further purification. All sugars employed were the natural forms.

Results. The data on comparative effect of a number of sugars and sugar derivatives on radiostrontium absorption and, in certain instances, on radiocalcium absorption are summarized in Table I. This compilation represents 3 separate experiments in which femur Sr^{85} values for control groups were standardized to a value of 1.3% for comparative purposes. However, results from each experiment were treated by analysis of variance method to determine any statistical significance of effects of test substances. First, there was parallel behavior between Ca^{45} and Sr^{85} in regard to effects on absorption, *e.g.*, xylose and lactose increased absorption of both alkaline earths whereas glucose and galactose had no effect on either ion. This parallelism holds in this laboratory for numerous substances, including some 20 amino acids and Vit. D(6,8,10).

Although radiostrontium responds qualitatively in the same way as does radiocalcium, there are certainly quantitative differences in over-all metabolism of these alkaline earths, a subject that has been studied intensively (11). Differences in the present study are shown by Sr^{85}/Ca^{45} ratios listed in Table I; here the Sr^{85}/Ca^{45} ratio of the ingested solution is taken as unity. The Sr^{85}/Ca^{45} ratio of 0.61 for the control group indicates that proportionally more Ca^{45} than Sr^{85} was transported from gut to bone. There was a definite increase in the Sr^{85}/Ca^{45} ratios of xylose and lactose-treated groups; this elevation of the Sr^{85}/Ca^{45} ratio by substances that en-

TABLE I. Influence of Carbohydrates on Gastrointestinal Absorption of Sr^{85} and Ca^{45} in the Rat.

Test substance	Femur conc. of radionuclide* (% dose/femur) Sr^{85}	Ca^{45}	Sr^{85}/Ca^{45}	Change in femur Sr^{85} content (%)	Effectiveness in enhancing Sr^{85} absorption (present study)	Effectiveness in enhancing Ca. retention (after Fournier *et al.*) †
Control	1.3 ± .1	1.9 ± .1	.61			
D-Glucose	1.1 ± .1	2.1 ± .2	.50	− 15	ineffective	ineffective
Sucrose	1.4 ± .1			+ 8	"	"
D-Galactose	1.5 ± .1	2.5 ± .2	.61	+ 15	"	slightly effective
D-Fructose	1.6 ± .2			+ 23	"	
Cellobiose	2.1 ± .2			+ 62	effective	
L-Sorbose	2.5 ± .1			+ 92	"	effective
D-Ribose	2.5 ± .2			"	"	
D-Xylose	2.6 ± .4	3.6 ± .3	.71	+100	"	effective
Lactose	2.6 ± .4	3.2 ± .2	.76	"	"	"
Raffinose	2.6 ± .2			"	"	"
Melibiose	2.6 ± .3			"	"	"
D-Glucosamine	2.7 ± .2			+108	"	
D-Mannitol	2.8 ± .2			+115	"	effective
D-Sorbitol	2.8 ± .3			"	"	"

* Values represent mean ± stand. error of mean; 6 rats/group; mean body wt about 90-110 g.

† See text for references.

hance alkaline earth absorption had been noted previously(6). The reason for this change in ratio is unknown but may be due to mass effects or the effect of these substances on processes of discrimination.

The relative effectiveness of test substances on Sr^{85} absorption may be readily noted in column 5 of Table I. The Sr^{85} contents in femurs of glucose, galactose, sucrose and fructose-treated groups were not significantly different from those of control group. All other sugars or their derivatives greatly enhanced Sr^{85} absorption, with cellobiose being least effective. Also in Table I, the data of Fournier *et al.* (*loc cit*) as obtained from the balance technic are summarized for comparison. There is excellent agreement between the 2 studies.

In Table II, results of the balance study with Sr^{85} are presented. The lactose-supplemented group absorbed and retained more of the ingested Sr^{85} than did the sucrose-supplemented group when measured directly or by bone content; these differences were significant at $p<0.01$. An important conclusion from this study is that the enhanced retention of Sr^{85} was accounted for by increased gastrointestinal absorption of Sr^{85}. This is shown by the similarity of "absorbed-retained" ratios for the 2 groups. Thus, there was no evidence that lactose had a direct effect on skeletal metabolism in these studies.

Discussion. From our study, it seems apparent that there was no general pattern of specificity in enhancement of Ca^{45} and Sr^{85} absorption by carbohydrates. Also there did not appear to be any correlation between effectiveness in promoting alkaline earth ab-

TABLE II. Comparison of Sucrose- and Lactose-Supplemented Diets on the Absorption and Retention of Radiostrontium.*

Diet	Fecal excretion of Sr^{85} (% ingested dose) †	Urinary excretion of Sr^{85} (% ingested dose) †	% absorption of Sr^{85} (by difference)	% retention of Sr^{85} (by difference)	Femur content of Sr^{85} (% ingested dose) †
Sucrose	78 ± 3	2.4 ± .3	22	20	.82 ± .09
Lactose	68 ± 2	4.4 ± .8	32	28	1.18 ± .04

* Basal diet contained casein 20%, starch 33%, vegetable oil 10%, brewer's yeast 2%, salt mixture U.S.P. #2 4%, vit. supplement (Nutritional Biochemical) 1%; sucrose and lactose added at 30%; 5 rats/group with mean initial body wt of 193 g.

† Values represent % of total ingested Sr^{85} that was excreted in feces and urine over the 7 day experimental period; data given as mean ± stand. error of mean.

sorption and any usual physical or chemical properties of the carbohydrate, which included pentoses, hexoses, disaccharides, trisaccharides, hexitols and hexosamines. This was in contrast to the specificity of the influence of individual amino acids on Ca^{45} and Sr^{85} absorption where L-lysine and L-arginine were more effective than any of 20 amino acids tested(6).

An interesting relationship may exist, however, between the physiological behavior of carbohydrates and their ability to enhance Sr^{85} absorption and calcium retention. Glucose and galactose are absorbed rapidly from the digestive tract and essentially by active metabolic processes; rate of absorption of glucose and galactose is decreased by presence of monoiodoacetic acid, phlorizin, an abnormal pH, and takes place at a constant rate, independent of concentration(12). Fructose absorption also occurs rapidly but takes place by 2 processes: simple diffusion and an active process that depends on transformation of intestinal fructose to glucose(12). The pentoses, sorbose and glucosamine are absorbed by passive diffusion as reported by Verzár(12) and Wilson and Vincent(13). Among disaccharides, lactose is absorbed more slowly than sucrose(14), the difference probably being due to the less rapid hydrolysis of lactose by intestinal enzymes. Cellobiose is absorbed much less rapidly by the rat than is glucose but can be utilized for glycogen synthesis(15). Information on comparative rates of absorption of sorbitol, mannitol, raffinose and melibiose or their comparative rates of enzymatic hydrolysis was not found. Thus, from information available, it appears that effectiveness in enhancing Sr^{85} absorption and calcium metabolism may be positively correlated with 2 physiological characteristics: (a) prolonged residence time of the effective carbohydrate in the gastrointestinal tract or (b) absorption by a passive mechanism. Although the exact meaning of this apparent relationship is not known, it is indicated from these studies that the site of action of effective carbohydrates is the gastrointestinal tract. This is in contrast to the theory of Fournier *et al.* (*loc cit*) that lactose, xylose, and other stimulatory sugars operate by directly influencing skeletal ossification.

Summary. 1. The influence of 14 carbohydrates on gastrointestinal absorption of radiostrontium and radiocalcium was studied in the rat. 2. Cellobiose, sorbose, ribose, xylose, lactose, raffinose, melibiose, glucosamine, mannitol and sorbitol significantly enhanced alkaline earth absorption whereas glucose, galactose, fructose and sucrose had little or no effect. 3. In a balance-type study with Sr^{85}, enhanced retention due to dietary lactose as compared with dietary sucrose could be accounted for by increased intestinal absorption rather than by direct effect on skeletal ossification. 4. Effectiveness in ability to enhance calcium and strontium absorption appeared to be positively correlated with a prolonged residence time of the carbohydrate in the gut or absorption of carbohydrate by a passive mechanism.

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Life of Guinea Pig Circulating Erythrocyte and its Relation to Erythrocyte Population of Bone Marrow.* (24923)

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In the course of quantitative studies of blood formation in the guinea pig(1,2,3) it became necessary to determine the life of the circulating red cell. The literature contains data on life span of red cells in 2 closely related forms, namely rat and rabbit, but no information about the guinea pig. In the rat Berlin and Lotz(4), after feeding glycine-2-C^{14}, estimated that the mean life span of the normal red cell was 64 days in the female and 68 days in the male. In the rabbit Neuberger and Niven(5), using glycine labeled with N^{15}, concluded that the mean life of the normal erythrocyte was about 65-70 days. In the present work we report efforts to determine the life of the circulating red cell in normal guinea pig by autoradiographic technic.

Technic. The work has been performed on 3 adult healthy male guinea pigs, of mixed strain, weighing approximately 400 g each. Each animal was given a single intraperitoneal injection of high specific activity† $Fe^{59}Cl_3$ (0.75 μc/g body weight), and blood smears were then made with blood obtained from ear vein from 12 hours to 8 days afterwards. The smears were fixed in methyl alcohol, then stained with haemalum and eosin. Coated radioautographs(6) were prepared using Eastman Kodak NTB-3 emulsion. The smears were finally stained differentially in 0.5% Giemsa solution at pH 6.4 for 12-15 hours at 5°C. Staining with either Giemsa or methylene blue before development is unsatisfactory, since methylene blue effects histochemical reduction of the emulsion(7). Preliminary staining with haemalum and eosin does not give rise to histochemical reduction and appears to improve subsequent Giemsa staining of cells through the emulsion.

Results. Radioautographs made of blood smears for the first several days after iron administration, provide for a clear separation between newly formed labeled erythrocytes and older non-labeled cells (Fig. 1). These labeled cells show a steady increase with time and at 7 days comprise 8.4% of the total (Fig. 2). Seven days, during which the rise is linear, is assumed to be the period in which there is enough Fe^{59} to label effectively all newly formed cells entering the blood stream. The radioautographs made at 8 or more days after Fe^{59} administration were not satisfactory for determining percent of labeled cells. This was due to reduced Fe^{59} content of cells entering the blood stream and thus the failure of single cells to produce distinct radioautographs. In the normal animal the level of circulating red cells represents a balance between newly formed cells and those undergoing destruction. If so, then in experimental animals in which 1.2% of newly formed red cells are daily entering the blood, the life of circulating red cells may be presumed to be $\frac{100}{1.2}$ *e.g.*, 83 days. This would make the life of guinea pig red cells appreciably shorter than that of the red cell in man, but longer than in the rat (64-68 days) or rabbit (65-70 days). It would be interesting to see whether application of other methods, to which reference has already been made, would confirm the figure thus obtained.

The significance of these data relates to the study of quantitative relationships between red cell populations of bone marrow and blood. Thus if one takes the data obtained in the Dunklin-Hartley strain of guinea pig(3,8) and assumes that the figures there given are applicable to our animals of mixed strain, then total circulating red cells are $157{,}766 \times 10^6$. If circulating red cells have a life of 83 days, then the daily requirement for newly formed red cells would be $1{,}900 \times 10^6$. The total nucleated red cell population of bone marrow is $2{,}616 \times 10^6$(3). This should

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† 1.3 mc/mg.

be quite capable of meeting the daily need for new red cells if the nucleated red cells of marrow have a mean doubling time of 33 hours. This is rather longer than the time arrived at in the previous calculation(3), based upon an estimated life of 70 days for circulating red cells, when it was concluded that the need of blood for new red cells would be met if the nucleated red cells of marrow had an overall doubling time of 28 hours.

The figure now obtained for the life of the circulating red cell, namely 83 days, makes the task of marrow rather less onerous than it would be on the basis of a life of 70 days. It should perhaps be noted that there is not a large reticulocyte reserve in guinea pig marrow(3) and therefore a need for additional red cells must in the main be met by increased formation of new cells. The extent to which the normal erythrogenic capacity is already taxed to its utmost is an important factor in considering the ability of marrow to cope with increased red cell formation.

Summary. Three male guinea pigs of mixed strain, and weighing approximately 400 g were given 0.75 μc of $Fe^{59}Cl_3$/g body weight and percentage of labeled cells appearing in blood was noted over 8 days. For the first 7 days there was a linear increase in percentage of labeled erythrocytes. On the assumption that this represents the normal rate of new red cell formation, it is calculated that the circulating red cell in the guinea pig has a life of 83 days.

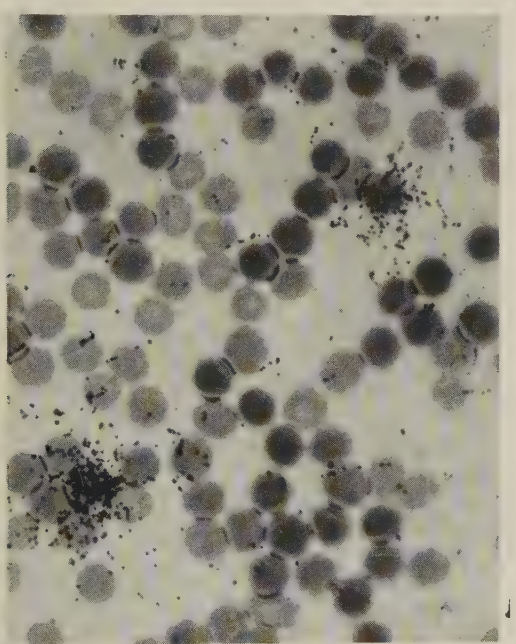

FIG. 1. Radioautograph of erythrocytes from blood smear made 24 hr after administering Fe^{59}. Two erythrocytes evidence characteristic labeling. 17 days' exposure. ×729.

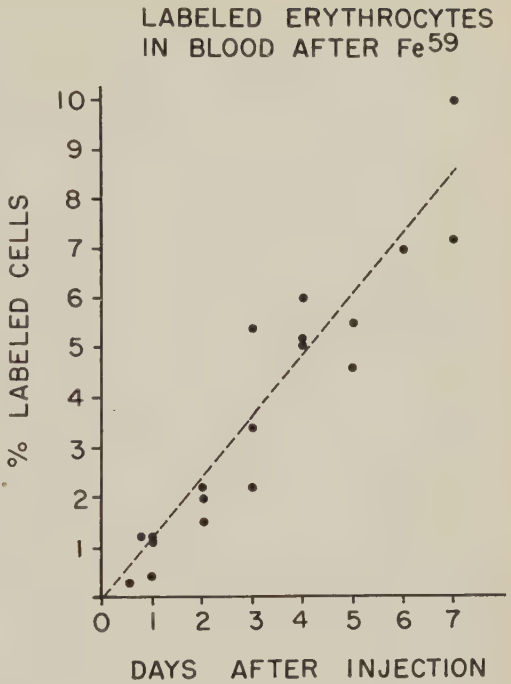

FIG. 2. Scattergram showing percentage of labeled erythrocytes in radioautographs of blood smears from individual animals at intervals after Fe^{59} administration.

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Factors Affecting Metabolism of Muscle Collagen. II. Effect of Radiation Exposure.* (24924)

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It has been shown(1) that turnover rates of both protein and its associated polysaccharide moiety of collagen are a function of age of rats studied. By use of glycine-2-C^{14} and glucose-U-C^{14} it was possible to show that the turnover rate of the protein component increased moderately with age, whereas that of the polysaccharide moiety decreased markedly. Using these experiments on normal animals as a basis for comparison, the present study deals with the effect of x-irradiation on collagen metabolism. Since radiation exposure appears to accelerate aging processes(2), a preliminary attempt has been made to determine whether or not x-irradiation simulates aging in its effect on metabolism of muscle collagen.

Methods. Two experiments were carried out using glycine-2-C^{14} (specific activity, 1.9 x 10^7 counts/minute (cpm)/mg and uniformly labeled glucose-U-C^{14} (specific activity, 2.5 x 10^7 cpm/mg). In each experiment, 2 groups of rats were used: one group was injected with glycine-2-C^{14}, the other with glucose-U-C^{14}. In the first experiment there were 4 animals/group; in the second experiment there were 6 animals/group. Total C^{14} dose stated in Table I was injected intraperitoneally in 3 equal doses on consecutive days. During period of isotope administration, rats used in first experiment were fed a synthetic diet (normal protein test diet fortified with vitamin supplements as obtained from Nutritional Biochemicals Corp.) whereas animals in second experiment were given Purina Fox Chow. The difference in protein content of the 2 diets probably accounts for the difference in specific C^{14}-activity of muscle collagen isolated in the 2 experiments involving glycine-2-C^{14} administration. Food and water were allowed *ad lib.* until 48 hours after last injection of C^{14}-labeled material. At this time, half the animals in each group were exposed to 756 r of 250 kv x-rays at 19.4 r/minute, using filters of 0.42 mm Al (parabolic) and 0.5 mm Cu. This dose represents approximately an LD 25 for 30-day survival. While test animals were exposed to x-rays, control rats were sham-irradiated. After irradiation, all animals were housed in individual cages without food until sacrificed 48 hours later. Collagen was extracted from skeletal muscle and purified according to the combined procedures of Neuberger *et al.*(3) and Fitch *et al.*(4) employed in that order. The hydroxyproline content of collagen so isolated was approximately 13%. Samples were plated as infinitely thin layers on polyethylene planchets and C^{14}-activity was determined using a gas flow counter in the Geiger region with a mixture of helium-isobutane as the quenching gas. The counting error in all instances was 3% or less. In the first experiment, each animal was processed individually, whereas in the second experiment, equal quantities of muscle from x-irradiated and sham-irradiated rats of each group were pooled separately prior to processing.

Results. Table I summarizes all pertinent data in both experiments. In experiments employing glycine-2-C^{14}, C^{14}-activity/gram collagen (or percent of injected dose) in irradiated rats is a little more than half the corresponding value for non-irradiated rats. This confirms an observation previously reported (6) using slightly different experimental conditions. When glucose-U-C^{14} was given, however, the reverse was observed, namely, C^{14}-activity/gram collagen in irradiated rats was almost twice that of controls.

Discussion. C^{14}-activity of glycine is incorporated into certain amino acid residues of the protein component of collagen, whereas

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TABLE I. Effect of Irradiation on C^{14}-Activity of Muscle Collagen.

No. of animals	Treatment	C^{14}-labeled compound inj.	Wt of animal (g)	C^{14}-activity inj./100 g body wt (cpm)	C^{14}-activity/g collagen (cpm)	% of inj. activity/g/collagen/100 g body wt
			Exp. 1			
1	X-irradiated	Glycine-2-C^{14}	199	2.2×10^6	7.9×10^3	.4
1	"	"	197	2.2 "	7.5 "	.3
1	Sham-irradiated	"	201	2.1 "	13.2 "	.6
1	"	"	203	2.1 "	13.0 "	.6
1	X-irradiated	Glucose-U-C^{14}	205	1.4 "	8.4 "	.6
1	"	"	206	" "	9.7 "	.7
1	Sham-irradiated	"	205	" "	5.7 "	.4
1	"	"	207	" "	5.9 "	.4
			Exp. 2			
3	X-irradiated	Glycine-2-C^{14}	164 ± 5	9.7×10^6	1.6×10^5	1.7
3	Sham-irradiated	"	166 ± 2	9.6 "	2.9 "	3.0
3	X-irradiated	Glucose-U-C^{14}	169 ± 2	4.4 "	21.3×10^3	.5
3	Sham-irradiated	"	167 ± 2	" "	9.1 "	.2

that of glucose contributes to the polysaccharide moiety(5). Therefore, irradiation caused a reduction in isotope concentration in the protein fraction of collagen and an increase in isotope concentration in the polysaccharide moiety as compared with control. From comparison with previous studies(5) of turnover rates of these components, it can be inferred that x-irradiation accelerates rate of turnover of the protein moiety and decelerates the rate of turnover of the polysaccharide component. In terms of its effect on metabolism of muscle collagen, one may conclude that exposure of rats to a dose of radiation in the lethal range produces acute changes similar to those occurring naturally as a result of aging.

Summary. 1. C^{14}-activity of muscle collagen was measured in x-irradiated and sham-irradiated rats given glycine-2-C^{14} and glucose-U-C^{14} prior to exposure. 2. Whole body x-irradiation resulted in reduction of isotope concentration of collagen in animals given glycine-2-C^{14} but caused an increase in isotope concentration when glucose-U-C^{14} had been administered. 3. It is concluded that x-irradiation and aging have similar over-all effects on collagen metabolism when studied with above-mentioned C^{14}-labeled precursors.

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Identification of *Neisseria gonorrhoeae* by Means of Fluorescent Antibodies. (24925)

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One of the more important applications of fluorescent antibody methods is pathogen identification in smears, without use of prior culture procedures(1,2,3,4,5). In the present work an attempt was made to develop similar technics for *Neisseria gonorrhoeae* identification because of their possible usefulness in control programs. Wilson(6,7) reviewed and studied antigenic relationships of gonococcus and meningococcus. It was demonstrated that 8 heat-stable antigens are shared by these species and that freshly isolated gonococcus cultures are frequently inagglutinable, but behave in a normal manner after being heated at 100°C for 30 minutes. In the same report it was also suggested that the "factor" responsible for inagglutination in freshly isolated cultures could be a substance similar to Vi antigen of *Salmonella typhi*. The antigenic properties of this "factor," however, were not demonstrated since rabbits inoculated with living inagglutinable cultures produced antibodies identical to those which received heat-killed cultures (Wilson, 1957, personal communication). Since surface antigens such as Vi(8) and the Kantigens of the Escherichia group(9) frequently control serologic activity of the bacterium, it is conceivable that the "factor" responsible for inagglutinable gonococcus cultures could be the most important diagnostic element of the *N. gonorrhoeae* cell. Identification of this material therefore has been of primary importance in the present work.

Materials and methods. Cultures of *N. gonorrhoeae* and other *Neisseria* were obtained from laboratory stocks and other laboratories.* Freshly isolated strains and smears were made available from the Division of Venereal Disease Control, Fulton Co. Health Dept., Atlanta, Ga. Cultures were grown on Eugon Agar(BBL)† plus 0.2% corn starch, and on GC Agar Base plus hemoglobin and Supplement B(Difco),‡ and were incubated in an atmosphere of 8-10% CO_2 gas at 35°C for 12-16 hours. Cultures were harvested in phosphate buffered saline pH 7.2 (BS) and killed by heating at 100°C for 30 minutes, 120°C for 2½ hours, and with 3% formalin for 30 minutes. Killed suspensions were washed 3 times with BS, adjusted to a density of either McFarland No. 10 or 10X McFarland No. 10. All suspensions were preserved by addition of 0.3% formalin. Live cultures were suspended in BS and used at density of approximately 20X No. 10 McFarland immediately after preparation. Antiserums were produced in healthy, young rabbits weighing approximately 5 lb each. Killed suspensions (McFarland No. 10 density) were injected *via* ear vein (IV) according to following schedule: 0.5 ml followed by 1 ml every 3-4 days for 2 weeks, one week rest followed by another 2 week injection schedule. One week after last injection, animals were bled from heart. Formalin-killed *N. gonorrhoeae* antigens were used in combination with Bacto-Adjuvant Complete (Difco). In this case, 5 ml of the antigen 5 X McFarland No. 10 was mixed with 5 ml of adjuvant. The mixture was administered by subcutaneous injection into the back of each animal. After one month these rabbits received additional IV injections as previously described, and were then bled. Antiserums were separated from blood clots in usual manner and stored at −20°C. Antigen suspensions for use in the slide agglutination tests were adjusted to density of 20X McFarland No. 10. Equal portions of undiluted serum and antigen were mixed on 1″ x 3″ microscope slides, hand-tilted or rotated, and observed against a dark

* Dr. Sara E. Branham's collection, Nat. Inst. Health, Bethesda, Md. Dr. Michael J. Pelczar, Jr., Univ. of Md., Baltimore, Md. Am. Type Culture Collection, Washington, D.C.

† Baltimore Biological Lab., Baltimore, Md.

‡ Difco Labs., Detroit, Mich.

background for clumping. Normal rabbit serums and BS were used as controls. Rabbit serum globulin was obtained by precipitation with ammonium sulfate. Fluorescein isothiocyanate (BBL) was employed in preparing globulin conjugates using a modification of the Goldman and Carver technic(10). Fluorescein isothiocyanate powder was added to chilled (4°C) globulin solution buffered at pH 9.0 (sodium bicarbonate-carbonate buffer), in the amount of .05 mg/mg protein. The mixture was shaken 6 hours in the cold, then dialyzed in BS to remove unreacted fluorescein. Where absorption of serums or conjugates is reported, packed cells were used in amount equal to volume of material to be treated. Cells were obtained and treated as described for antigens. Absorptions were carried out for 4 hours at 50°C. Cells were removed by centrifugation at 12,800 x G for one hour in the cold. Smears prepared for microscopic observation were made from antigens (prepared as previously described) and from 16 hour living cultures. Smear fixation was accomplished by air drying or by light heat. Undiluted fluorescein conjugates (1 g % protein) were used for staining. Unabsorbed conjugates were applied to smears for 30 minutes at 37°C, using rotation as described earlier(11). Staining times were frequently increased to one hour, as necessary, when absorbed conjugates were used. After staining, smears were washed in running BS and allowed to stand in same solution for 30 minutes. Slides were finally blotted lightly and a small drop of mounting mixture (9 parts C.P. glycerine plus 1 part BS) was placed on each smear. A cover glass placed upon the mounting mixture completed the slide preparation. Observations were made using Leitz or Reichert microscopes equipped with appropriate filters and ultraviolet light assemblies. Photomicrographs were recorded on 35 mm Super Anscochrome, daylight film. Fluorescent antibody controls included normal rabbit globulin conjugates and inhibition of specific staining by means of unlabeled globulins.

Results. Agglutination findings (Table I) confirm the observations of Wilson that gonococcal cultures may be inagglutinable in homologous antiserums. Since formalin-killed cultures, represented by GC(F), demonstrate inagglutinability like that of the living culture, GC(L), it appears that formalin preserved the characteristic of the substance or "factor" responsible for inagglutination. Since the antiserum produced against formalin-killed antigen GC(F) agglutinates all antigens (L, F, 100° and 120°) while GC (100°) serum clearly agglutinates only antigens GC (100°) and (120°), it would seem that an antigen has been preserved by formalin treatment, making GC(F) similar to GC(L). However, since all reactivity was removed from GC(F) serum by absorption with antigen GC(100°), it appears that this antigen must also contain some of the same "factor." If this "factor" is similar to the K antigens of the Escherichia group, then the reaction noted would indicate that it is of the B type (agglutination characteristics destroyed by 100°C, absorption ability, unaltered). This antigen, however, is apparently destroyed at 120°C because antiserum produced by GC(F) after being absorbed with antigen GC(120°) contains agglutinins for L and F antigens, but does not contain agglutinins for 120°

TABLE I. Slide Agglutinations Employing *N. gonorrhoeae* Antigens and Antiserums.

	Serums undiluted					
Antigens*	GC (F)	GC (100°)	GC (120°)	GC (F) absorbed with GC (120°)	GC (100°) absorbed with GC (120°)	GC (F) absorbed with GC (100°)
GC (L)	+	±	—	+	±	—
" (F)	+	±	—	+	±	—
" (100°)	+	+	+	+	+	—
" (120°)	+	+	+	—	—	—

* Representing 5 strains.

L = Living culture; F = Formalin killed; 100° = Heat killed; 120° = Heat killed; —, ±, + = Degrees of agglutination.

TABLE II. Neisseria Reactions with *N. gonorrhoeae*—GC (F) Fluorescein Labeled Antiserum.

Antigens	Unabsorbed	Single Absorption with serogroup A (100°)	Single Absorption with serogroup A (F)
Gonococcus			
GC (L) (3 strains)	*3+ s oc 4+ s	3+ s oc 4+ s	3+ s oc 4+ s
(100°)	3+ oc 4+	3+ oc 4+	3+ oc 4+
Urethral smears (25 individuals)	4+ s	4+ s	4+ s
Meningococcus			
Serogroup A (L)	1+ oc 4+ s	— oc 3+	—
(100°)	1+ oc 4+ s	1+	—
Serogroup B (L)	1+ oc 4+ s	1+	—
(100°)	1+ oc 3+ s	1+	—
Serogroup C (L)	1+	1+	1+
(100°)	2+	1+	—
N. catarrhalis (L)	1+	1+	1+
(100°)	1+	1+	1+
N. perflava (L)	—	—	—
(100°)	—	—	—
N. sicca (L)	—	—	—
(100°)	—	—	—

* Degrees of fluorescents: —, 1+, 2+, 3+, 4+ (1+ barely visible). s = Solid stain. oc = Occasional.

antigens. Thus it would appear that the 120° antigen represents a somatic complex, or the O antigens comprised by the gonococcus. The K-like antigen of the gonococcus will hereafter be referred to as the GC-K(B).

Table II gives fluorescent antibody reactions obtained by staining various *Neisseria* antigens with absorbed and unabsorbed GC (F) fluorescein labeled antiserum. Strong cross-reactions or staining occurred when unabsorbed conjugate was used to stain meningococcal Serogroups A and B. However, since the somatic antigens of gonococcus and meningococcus are shared(7,8), the fluorescent antibody reactions were interpreted as resulting from this relationship. Minor reactions will also be noted for Serogroup C and *N. catarrhalis*. A single absorption with Serogroup A (100°) and A (F) antigens removed all cross-reactions of a troublesome nature, yet leaves an adequate supply of specific antibody for gonococcus identification in smear preparations.

Typical gonococcus fluorescent reactions are illustrated in Figs. 1, 2 and 3. Organisms in urethral smears are brilliant and solid in appearance, while young inagglutinable cultures lose some of the solid staining character. Older cultures, Fig. 3, show poor staining, indicating K antigen loss.

Discussion. Our evidence appears to indicate that *N. gonorrhoeae* possesses a heretofore unrecognized antigenic component similar in character to Vi antigen of *S. typhi* or K antigen of the Escherichia group. The new antigen appears to be species specific and may be recognized by slide agglutination tests employing living or formalin-killed cultures and by fluorescent antibody technics. The fluorescent antibody method for *Neisseria* study allows detection of serologic reactions which are not apparent by the usual agglutination tests. It appears to be an extremely valuable tool in determining development and/or loss of particular antigens of the gonococcus in relation to nutritional and physical growth conditions. It is evident that further studies are justified in respect to production of species specific antiserums of high titer, of determining additional *Neisseria* relationships based on absorption technics and demonstration of strain or type antigenic differences in *N. gonorrhoeae* cultures.

Summary. 1. A species specific antigen, associated with freshly isolated, inagglutinable gonococcus cultures, is recognized. 2. The new antigen appears to possess characteristics similar to Vi antigen of *S. typhi* or the K an-

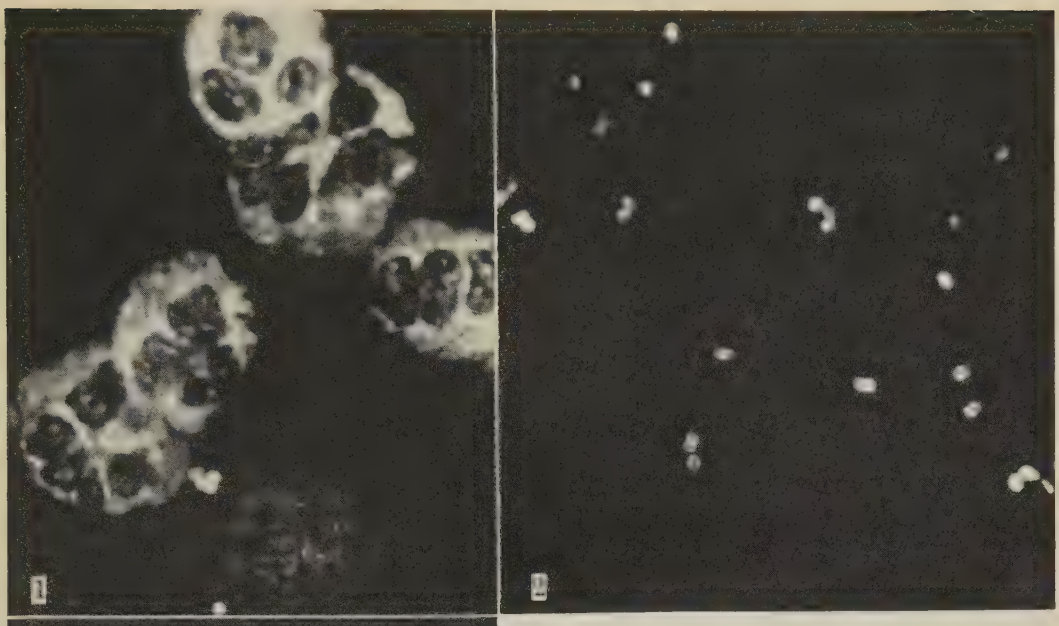

FIG. 1. Urethral (male) smear stained with GC(F), fluorescein labeled antiserum. Solid staining of gonococci indicates fully developed GC-K (B) antigen.

FIG. 2. Young culture (12-16 hr) stained as previously described. GC-K(B) antigen well developed but not as complete as in Fig. 1.

FIG. 3. Older culture (30 hr) illustrating extreme lability of GC-K(B) antigen. Some cells show only a remaining trace of the substance.

tigens of the Escherichia group, and is fully developed only in freshly isolated cultures or in infectious exudate. 3. Antiserums containing GC-K(B) antibodies may be labeled with fluorescein and used as a means of identifying *N. gonorrhoeae* in smear preparations.

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Effect of Intestinal Tract Irradiation on Serum Proteins of the Rat.* (24926)

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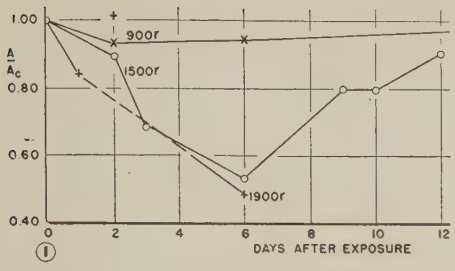
Several authors have reported that protein composition of rat serum is altered after whole-body x-irradiation(1,2,3). When extensive intestinal tract irradiation studies were initiated in this laboratory(4), it was felt that a study of effects of various types and methods of intestinal tract irradiation on serum proteins might shed light on the mechanism of radiation damage to intestinal tract. This report describes results obtained by paper electrophoretic fractionation of proteins in sera of animals at various intervals following x-irradiation of whole body, or intestinal tract, both *in situ* and exteriorized. Results are also included from animals that received beta irradiation of the GI tract from administration of Y^{91} by stomach tube, and from animals maintained on starvation diet to simulate decreased food consumption of irradiated animals.

Methods. Animals used were adult female Sprague-Dawley rats weighing 200 to 240 g. Twelve animals were exposed to 600 r of whole-body x-irradiation. The radiation factors were 250 kvp, 30 ma, HVL 1.15 mm Cu, TSD 96 cm and dose rate was about 35 r/min as measured in air with Victoreen ionization thimbles. Seventy-four animals were exposed to x-irradiation of gastrointestinal tract, which was irradiated either *in situ,* or surgically exteriorized, while remainder of body was protected by lead shielding. Intestines of rats, under Nembutal anesthesia (30 mg/kg), were exteriorized through midline incision by gentle manipulation and exposed to radiation on cardboard plaque. The remainder of body was incased in one-fourth inch thick, cylindrical, half shell-type lead shield(4). The intestine was moistened with Ringer's solution and maintained at body temperature throughout exposure. Asepsis was practiced in all surgical and exposure manipulations. After irradiation the GI tract was replaced, the abdominal wall was sutured and skin closure was made with skin clips. Rats exposed to abdominal irradiation were placed on their side while under anesthesia. Shields for these exposures were constructed of one-fourth inch lead with aperture approximately 3 x 5 cm (4). Region receiving direct irradiation extended anteriorally about 3 cm from greater trochanter and posterior to rib cage. The vertebral column, spleen, kidneys and almost the entire liver were shielded from direct radiation. Surgical and/or normal control animals were run in parallel with irradiated animals. Radiation factors were as above. Nineteen animals received 3 cm of Y^{91} by stomach tube as yttrium chloride at pH 3. Control rats were administered an equal volume of pH 3 hydrochloric acid. Starvation control animals were deprived of food for 3 days, then maintained on 50% of normal diet for additional 6 days. Blood samples were obtained from tail vein for serial determinations and by cardiac puncture, when animals were to be sacrificed. Electrophoretic characterization of serum samples was done by the Durrum method (5) using Veronal buffer at pH 8.6 and 0.1 ionic strength. Protein composition of serum was determined by scanning dyed filter paper strips in Spinco Analytrol Photometer-Computer.

Results. In all groups of irradiated animals per cent albumin in sera showed largest and most consistent changes following irradiation. This consistency was probably due to better precision of interpreting the "Analytrol" trace for per cent albumin than for other serum protein fractions. To present dose dependency of composition of serum proteins as clearly as possible, the percent albumin in irradiated animal sera was divided by average of per cent albumin in control animal sera fractionated in the same electrophoresis "run." This quotient, expressed as A/A_c, tends to minimize day to day variations in electrophoresis procedures. It is recognized that this electrophoretically separated "albumin" fraction

* This work was performed under Contract between Atomic Energy Comm. and General Electric Co.



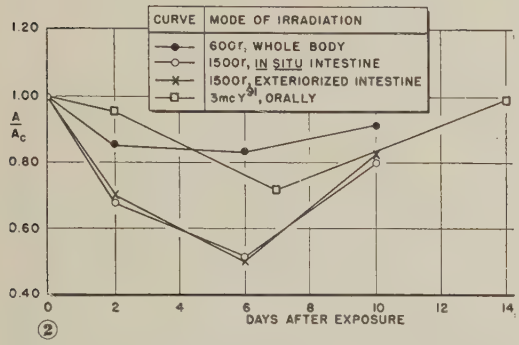


FIG. 1. Effect of *in situ* intestinal x-irradiation on A/A_c value in rat serum.

FIG. 2. Effects of various modes of irradiation on A/A_c value in rat serum.

may have a complex composition(6,7).

Fig. 1 shows variation with time and dose of average A/A_c values of sera from rats whose intestinal tracts were irradiated *in situ*. Each point is the average of results from 3 to 9 animals. The reason for the apparent discrepancy in results from one- and 2-day animals that received 1,900 r is not known. Fig. 2 compares effects of different modes of irradiation of comparable acute lethality on the A/A_c values obtained at different times after exposure.

Discussion. Maximum depression in albumin level was observed 6 days after x-irradiation (Fig. 1). Time of maximum depression and pattern of subsequent recovery are similar to the effect of intestinal irradiation on other blood elements(4). Amount of histological damage and time of maximum mortality caused by acute doses of radiation to the intestine suggest a relationship among these effects.

Although decreased albumin level was caused by exposure of abdominal region, the possibility remained that the liver may have absorbed sufficient "scatter radiation" to cause inhibition of serum albumin synthesis at this site. Therefore, serum protein pattern was determined in animals totally shielded except for an exteriorized portion of intestinal tract which was irradiated. The decrease in serum albumin was approximately the same, whether the gut was irradiated, exteriorized or *in situ* (Fig. 2). This effect must therefore be due either to direct action of radiation on the intestine, or to indirect action by some material produced in the gut during irradiation acting at site of serum protein synthesis.

Fig. 2 shows the effect on serum albumin of internal beta irradiation of the GI tract. The 3 mc dose of Y^{91} administered is of comparable acute lethality to 1,500 r of x-ray. Since yttrium is absorbed from the GI tract to a negligible extent, its effect must be attributed to irradiation of intestines and closely adjacent tissues. Albumin is decreased following oral administration of Y^{91}, but onset of this effect is delayed, and the magnitude decreased, as compared with x-radiation effects. This is probably related to the longer time required for intestine to absorb a beta-radiation dose comparable to the x-ray dose.

Exposure of rats to whole-body x-radiation of comparable acute lethality (600 r) caused a much smaller effect on serum albumin than did the 1,500 r dose to the intestinal tract. Maintaining animals for 3 days without food, then for 6 days on only 50% of their normal diet had no effect on A/A_c values measured.

Summary. The results show that irradiation of the gastrointestinal tract affects albumin content of rat serum in the same manner as does whole body irradiation. This effect has been attributed to irradiation of the liver during whole body exposure. Since only the intestinal tract was irradiated in these experiments, decreased albumin production caused by indirect action of some substance originating in the irradiated tissue is suggested. Another possibility is loss of albumin because of increased capillary permeability in the irradiated intestinal tract.

The authors wish to acknowledge able technical assistance of Alma L. Crosby and John T. Homer.

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Growth and S^{35} Uptake of Cotton Granulomas in Rats. (24927)

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The cotton pellet granuloma technic of Meier, *et al.*(1), has provided a new approach to studies of inflammatory potentials of animals under various physiological conditions(2-5). In addition it has provided the basis for development of several tests for anti-inflammatory materials(6-11). This is a study of long term growth of granulomas around cotton pellets and incorporation of labeled sulfate into the tissue in early stages of granuloma formation.

*Methods.** Male Sprague-Dawley rats averaging 175-200 g in weight were adrenalectomized 2 weeks after arrival and maintained on saline and Purina laboratory chow. The saline was supplemented with 0.5% glucose for 24 hours after adrenalectomy. In an experiment designed to study granuloma growth and histology, 4 cotton pellets averaging 6 mg in weight were implanted subcutaneously(11) into each rat the day following adrenalectomy, and the granulomas were removed at autopsy 2-100 days later. In a second experiment in which sulfate uptake in granuloma tissue was measured, each rat was implanted with a 6 mg pellet 12, 6, 4 and 2 days before autopsy. These animals were adrenalectomized on day before first pellet was implanted, and placement of pellets was varied to allow for possible position effects. Twenty-four hours before sacrifice all animals received 150 microcuries of S^{35} labeled sodium sulfate subcutaneously. In both experiments test compounds were administered subcutaneously as solutions or suspensions in oil beginning first day of implantation. At autopsy animals were sacrificed with ether, the granulomas removed, and those granulomas used for growth curve or S^{35} studies were trimmed of extraneous connective tissue. Granulomas used for histological purposes were fixed in Zenker's solution untrimmed. As control tissue for the S^{35} data normal connective tissue was removed from subcutaneous area of back according to method of Boas and Foley(12). Trimmed granulomas and normal connective tissue were dried to constant weight at 72°C. Granuloma weights were expressed as total granuloma weight/rat by subtracting original weight of the 4 cotton pellets. For radioactivity measurement individual granulomas and tissue samples were weighed and wet-ashed in perchloric acid. Aliquots of each tissue digest were mounted directly on lens paper discs supported by copper planchets and counted with end-window Geiger counter. In calculating percentage of administered S^{35} incorporated/g of tissue, corrections were made for self-absorption, radioactive decay, and variations in body weights of animals. Granulomas for histological examination were fixed in Zenker's solution 24 hours, then cut in half and washed in tap water. They were dehydrated with S-39 Technicon dehydrant, cleared with butyl acetate, infiltrated 3 or 4 days in paraffin and embedded. The paraffin blocks were trimmed to the tissue and soaked in detergent for several days to soften the cotton fibers for cutting. Chilled blocks

* The suggestions and encouragement by Dr. R. C. Kelsey on sectioning granulomas are gratefully acknowledged.

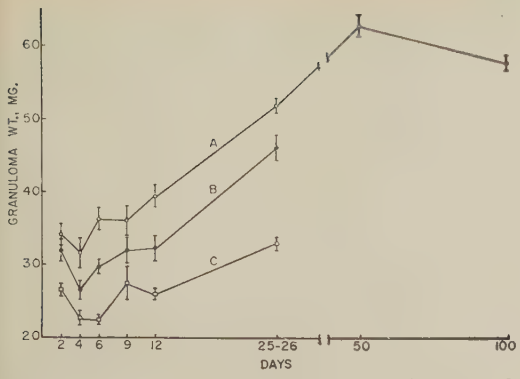

FIG. 1. Growth of cotton granulomas in adrenalectomized rats treated with oil (A), or cortisol at .25 mg (B) and 1 mg (C) per day. Vertical bars show stand. errors of means.

were cut at 6 μ, and sections stained with hematoxylin and eosin or Mallory's stain.

Results. Fig. 1 shows that cotton pellet granulomas increased in weight for 50 days after implantation. Rate of growth decreased after 25 days, and granuloma weights either remained constant or fell off between 50 and 100 days. The apparent fall in granuloma weight at 4 days was not statistically significant ($P>.05$). Daily cortisol treatment retarded granuloma growth markedly but did not completely block growth at dose levels used (Table I).

Histological studies of early granulation tissue encapsulating 2 day pellets showed many phagocytes, polymorphonuclear neutrophils, and immature connective tissue cells, while few mature fibroblasts and collagen fibers were seen. Little tissue grew into the cotton pellet although many degenerating polymorphs were observed among the cotton fibers. Granulomas encapsulating pellets implanted for 6 days showed more fibroblasts and fibers than those implanted for 2 days although many macrophages were still present. At the 6 day stage macrophages and fibroblasts started to penetrate into the pellet between cotton fibers while degenerating polymorphs were still found in center of pellet. The capsules of 25 day granulomas were dense with collagen fibers and elongated fibroblasts. These elements, vascular channels, and some macrophages penetrated all the way to a small tissue-free region at center of pellet. Granulomas of 50 and 100 days of age were somewhat more fibrous than younger tissues; and in addition, granulation tissue had penetrated throughout the center of pellet. Grossly, all granulomas were approximately the same diameter with later growth progressing to center of pellet rather than peripherally.

Incorporation of S^{35} into 4, 6 and 12 day granulomas was significantly higher ($P<.01$) than that exhibited by normal connective tissue (Fig. 2). Cortisol treatment, however, significantly decreased the labeled sulfate incorporation into normal tissue as well as into granulomas of all ages ($P<.01$). Similarly, phenylbutazone decreased S^{35} uptake in the granuloma tissue ($P<.01$ at 2, 4, and 6 day; $P<.05$ at 12 day); but its effect on normal connective tissue was negligible ($P>.05$). 17α-ethyl-19-nortestosterone (Nilevar) exhibited no effect upon sulfate incorporation into either normal tissue or granulomas.

Discussion. In the present report we assumed that S^{35} found in tissues studied was present primarily as sulfated mucopolysaccharides(13). As granulomas aged, rate of incorporation of S^{35} into these mucopolysaccharides exceeded rate of tissue growth. Thus, control granuloma weights increased but 5% between 2 and 6 days (Table I), while the

TABLE I. Growth of Cotton Granulomas in Adrenalectomized Rats.

	Oil control		Cortisol, 0.25 mg/day		Cortisol, 1 mg/day	
Days	Rats	Granuloma wt, mg	Rats	Granuloma wt, mg	Rats	Granuloma wt, mg
2	41	34.1	41	31.9	41	26.6
4	20	31.7	20	26.5	20	22.7
6	26	36.2	26	29.7	26	22.4
9	12	36.0	12	32.0	12	27.4
12	27	39.4	7	32.3	7	25.9
25–26	37	52.0	14	46.2	16	33.1
50	20	63.0				
100	19	58.1				

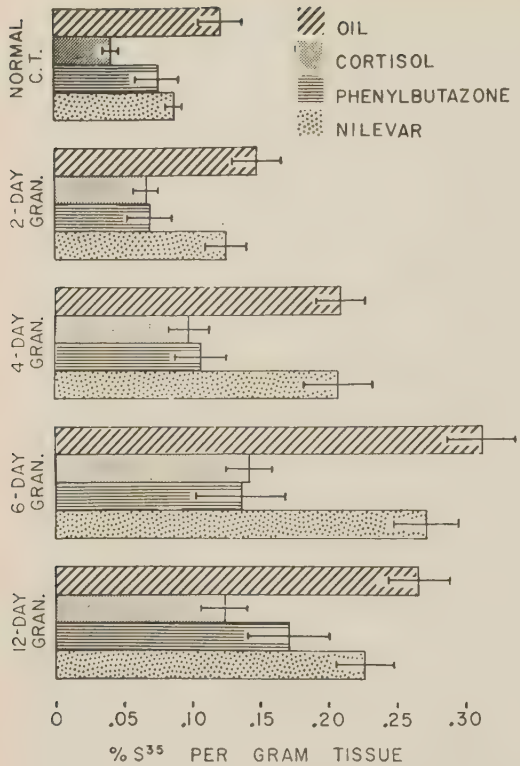

FIG. 2. Incorporation of labeled sulfate into normal connective tissue and cotton granulomas in adrenalectomized rats. Rats (11-15/group) were treated daily with oil; cortisol, 1 mg; phenylbutazone, 20 mg; or Nilevar, 1 mg; for 12 days. Small bars represent stand. errors of means.

amount of S^{35} incorporation into this tissue nearly doubled in the same time (Fig. 2). This coincided with a shift in cell population from undifferentiated cells, polymorphs, and macrophages to a greater proportion of fibroblasts as well as increase in collagen fibers. The inhibitory effect of cortisol on S^{35} uptake was consistent with the observation that anti-inflammatory corticoids inhibit synthesis of chondroitin sulfate(14). Seemingly, phenylbutazone was more active in inhibiting incorporation of S^{35} into forming granuloma mucopolysaccharide than in affecting normal tissue metabolism. While we found negligible effects of 17α-ethyl-19-nortestosterone upon S^{35} uptake in granuloma tissue, Kowalewski and Morrison found this substance to enhance incorporation of labeled sulfate into bone mucopolysaccharide(15) suggesting that 17α-ethyl-19-nortestosterone may have a greater effect upon S^{35} incorporation in bone than in granuloma tissue.

Summary. 1) Cotton pellets implanted subcutaneously in rats induced growth of foreign body granulomas that did not completely penetrate the pellet or reach maximal weight until 50 days. As granulomas aged, shifts in connective tissue elements coincided with increases in uptake of labeled sulfate. 2) Cortisol treatment decreased S^{35} incorporation into both granuloma and normal connective tissue. Phenylbutazone reduced labeled sulfate uptake by granulomas, but not normal connective tissue, while 17α-ethyl-19-nortestosterone had no effect on either.

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Effect of Hyperthyroidism on Distribution of Adenosine Phosphates and Glycogen in Liver.*† (24928)

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The experiments described below were undertaken with two objectives. The first derives from the postulate that the mechanism of action of the thyroid hormone consists of a dissociation of phosphorylation from oxidation, in a manner analogous to the effect of dinitrophenol. Such a dissociation was found by Maley and Lardy(1) in lowering the P:O ratio of isolated liver mitochondria from thyrotoxic rats. The *in vivo* equivalent might be expected to manifest itself as a shift in the balance between ATP and the products of dephosphorylation, ADP and AMP, under steady state conditions in the intact animal. The second objective was as a test of the hypothesis of Bloom *et al.*(2) that the fraction of liver glycogen which can be extracted by cold TCA§ is more active metabolically than the fraction which is not so extractable, which can be isolated by treatment with hot KOH solution.

Methods. Experiments were carried out on guinea pigs because of their sensitivity to thyroxin. Males weighing at least 500 g were maintained on Purina guinea pig chow and water *ad lib.* Oxygen consumption under conditions approximating basal was determined by modification of the method of Watts and Gourley(3), using a large vacuum type desiccator as the animal chamber. When a number of consistent readings were obtained over several days, the experimental animals were given daily injections of L-thyroxin subcutaneously, in dose of 100 μg/kg. The thyroxin was dissolved in minimum volume of 0.1 *N* NaOH, and diluted with 0.9% solution of NaCl. Two weeks of this treatment usually increased oxygen consumption by at least 50%. A few animals were more refractory and required a daily dose of 150 μg/kg. One normal and one thyrotoxic animal were worked up simultaneously. Under pentobarbital anesthesia, supplemented by ether when necessary, the liver was excised and dropped immediately into a large volume of freezing mixture of dry ice and ether. Subsequent operations were carried out in cold room at 1-2°C. The frozen liver was broken into small pieces in a tissue crusher chilled with dry ice. A weighed quantity was transferred to a Waring blendor, and treated with approximately 5 volumes of 10% solution of TCA. The blendor was run for about 2 minutes, to obtain complete precipitation of proteins. The suspension was centrifuged, the supernatant filtered, and the residue extracted twice with 5% TCA solution. The combined filtrate and washings were extracted with ether to remove the TCA, the solution made ammoniacal, and brought up to definite volume. An aliquot of this was taken for precipitation of glycogen by ethanol. The bulk of the solution was then passed over a column of Dowex-1 resin to adsorb the P compounds present. The column was washed with water, then with a liter of 0.01 *M* NH_4Cl. The sequence of eluents used was: 0.002 *N* HCl, which removed AMP; 0.01 *N* HCl, which removed first some nucleotide matter containing bases other than adenine, and then ADP; 0.01 *N* HCl plus 0.04 *M* KCl, which eluted additional nucleotide compounds with other bases than adenine; and finally with 0.02 *N* HCl plus 0.6 *M* KCl, to elute ATP. Elution was at a rate of approximately 50 ml/hour, and 2-hour fractions were collected by automatic fraction collector. Progress of the elution was followed by measurements at 260 and 280 mμ in a Beckman

* This work was carried out under Contract between U. S. Atomic Energy Comm. and Univ. of Arkansas.

† The material presented here is taken from thesis submitted by Oscar P. Chilson in partial fulfillment of requirements for degree of Master of Science.

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§ Abbreviations used are: TCA for trichloroacetic acid, and ATP, ADP, and AMP, for adenosine tri-, di-, and mono-phosphates, respectively.

TABLE I. Effect of Hyperthyroidism Produced by Thyroxin on Adenosine Phosphates and Glycogen of Liver in Guinea Pigs.

	AMP	ADP	ATP	TCA-extractable glycogen, mg/g	Non-extractable glycogen, mg/g
	(μM/g)				
Normal (7 exp.)	.945 ± .25	1.49 ± .17	.99 ± .09	31.4 ± 1.7	3.6 ± .6
Hyperthyroid (7 exp.)	.77 ± .19	1.82 ± .10	.86 ± .11	1.9 ± .3	3.4 ± .6

Values as mean and stand. error of mean.

Model DU spectrophotometer. The residue from the TCA extraction was digested with 30% KOH solution and the glycogen isolated by precipitation with ethanol. The material was dissolved in water, proteinaceous material precipitated by addition of TCA, and the glycogen re-precipitated by ethanol. Glycogen determinations were made by the anthrone method(4) using purified glycogen isolated from rabbit liver as a standard.

Results obtained are given in Table I. It is evident that ATP comprises less than one-third of total adenosine phosphates present in liver in water-soluble forms, and that ADP is the form present in highest concentration. This distribution makes it possible to ascertain whether there is any shift in the balance in the thyrotoxic state. Neither with respect to total adenosine phosphates nor to distribution between ATP, ADP and AMP, was any significant difference found between normal and thyrotoxic animals. These data, therefore, lend no support to the theory that thyroid hormone produces a dissociation of phosphorylation from oxidation. However, there is still the possibility that such a shift in the balance between ATP and the dephosphorylation products might be obtained acutely, as a result of a single injection of a sufficiently large dose of tri-iodothyronine.

The decrease in glycogen content of the liver, which is well known as an effect of the hyperthyroid state, is clearly seen to be limited to that fraction which is extractable by TCA. This finding strengthens the hypothesis of Bloom *et al.*(2) that TCA-extractable glycogen is more active metabolically than the fraction which cannot be extracted from the liver by this means.

Summary. Chronic hyperthyroidism induced in guinea pigs by daily injection of thyroxin has no effect on concentrations of ATP, ADP, and AMP in the liver. This finding gives no support to the postulate that thyroxine produces its metabolic effects by dissociating phosphorylation from oxidation. Reduction in glycogen content of liver that results from chronic hyperthyroidism is entirely in the fraction extractable by TCA.

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Effect of Reserpine on Oxytocin and Lactogen Discharge in Lactating Rats.* (24929)

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It is generally accepted that oxytocin as well as lactogen are reflexly released from the hypophysis in lactating animals and that both hormones are important factors in determin-

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† Postdoctoral Fellow of N.I.H. This investigation supported in part by grants from P.H.S.

ing amount of milk obtained during nursing or milking. Although lactogen is essential for initiation and maintenance of lactation(1), oxytocin causes contraction of the myoepithelial elements(2) surrounding mammary alveoli and small ducts thus forcing milk into larger ducts where it is available for withdrawal. Recent investigations have indicated reserpine may stimulate production and release of lactogenic hormone(3-5) but may depress that of thyrotropin(6), gonadotropin (7) and, under certain conditions, adrenocorticotropin(8). Little is known, however, of the effect of the drug upon oxytocin release. In the present study we have determined the effect of reserpine upon oxytocin discharge as evaluated by the milk let-down method of Grosvenor and Turner(9). We have also determined lactogenic hormone content of the pituitary of 14-day postpartum lactating rats immediately prior to and following 30 minutes nursing.

Material and method. Seventy-two adult primiparous lactating rats of the Sprague-Dawley-Rolfsmeyer strain weighing 260-320 g were housed in individual cages and allowed free access to food and water. Shortly after parturition, each litter was reduced to 6 young and when 14 days old was isolated from their mother for 10 hours. During the isolation period, the mothers were treated as follows: (a) 39 received single subcutaneous injections of reserpine‡ in dose of 10 μg/100 g either 10 or 120 minutes prior to replacement of litters. (b) 8 received single subcutaneous injections of reserpine (10 μg/100 g) 120 minutes prior to replacement of litters followed by .2 USP/kg oxytocin 2 minutes prior to replacement of litters. (c) 25 served as controls and were uninjected. At end of isolation period, 15 reserpine-treated and 10 control mothers were killed with ether and their pituitaries rapidly removed, weighed individually and frozen until assayed for lactogenic hormone. The remainder were reunited with their litters and allowed to nurse for exactly 30 minutes. Immediately after nursing the mothers were killed, their pituitaries removed and prepared as above. The litters were then weighed, killed by decapitation and stomach contents removed and weighed. Quantity of milk obtained by litters of lactating rats in 30 minutes, expressed as percent litter body weight, was used as index of milk let-down activity. Four or 5 pituitary glands were pooled for each lactogenic hormone assay. These were crushed in an agate mortar, suspended in a measured amount of distilled water and assayed in adult common pigeons by the micromethod of Grosvenor and Turner(10). Solutions of standard lactogenic hormone§ were run simultaneously in some assays. Average crop gland responses were converted to mg lactogenic hormone by use of the regression equation from the assay procedure(10) since the average response to the standard was 5-10% of that produced with the corresponding dose of the standard in the original assay. Gross observations were also made on the effect of reserpine on behavior of the animals.

Results. Rats treated with reserpine did not exhibit ptosis or any external signs of sedation. Nursing behavior appeared normal for upon replacement of the litters the mothers would retrieve their young and begin nursing within 1-4 minutes. Amount of milk obtained by litters of lactating rats injected with reserpine 120 minutes prior to litter replacement was significantly less than that obtained by control offspring ($P<.001$). Although the quantity of milk obtained by young of mothers treated 10 minutes before nursing was significantly less ($P<.05$) than that obtained by control young, the range of values was greater than that of animals treated 120 minutes before litter replacement as evidenced by the larger standard error (Table I). When oxytocin was injected after reserpine, significantly larger ($P<.03$) quantities of milk were obtained by offspring in comparison with control group. Pituitary lactogenic hormone concentration of control mothers killed at end of 10 hour isolation period was 122.1 I.U./g pituitary (prenursing level). Following 30 minutes nursing, the hormone content had decreased approximately

‡ Kindly supplied by Ciba Pharmaceutical Products and Eli Lilly and Co.

§ Highly purified sheep preparation estimated to contain 20 I.U./mg.

TABLE I. Effect of Reserpine on Quantity of Milk Obtained in 30 Minutes by Litters of 14-Day Postpartum Lactating Rats following 10 Hours Isolation of Mother and Litter.

Treatment	No. of rats	Time of treatment prior to nursing (min.)	Avg wt (g) of: Litters	Milk	Avg % Milk wt / Litter wt
Control	15		186.4	7.87	4.25 ± .31*
Reserpine, 10 μg/100 g	10	10	169.5	5.26	3.09 ± .41
Idem	14	120	174.6	4.02	2.28 ± .22
" , then oxytocin, .2 USP/kg	8	2	161.3	9.84	5.51 ± .32

* Stand. error of mean.

87% to a postnursing level of 16.4 I.U./g. Administration of reserpine 120 minutes prior to litter replacement decreased the prenursing level 48% (64.1 I.U./g) when compared with the control level (Fig. 1). The postnursing level of reserpine-treated animals was 23% (12.7 I.U./g) lower than the control value.

Discussion. Reserpine has been shown to exert an inhibitory effect upon the ovary, thyroid and adrenal gland(6-8) presumably through suppression of neural centers within the hypothalamus and/or reticular formation

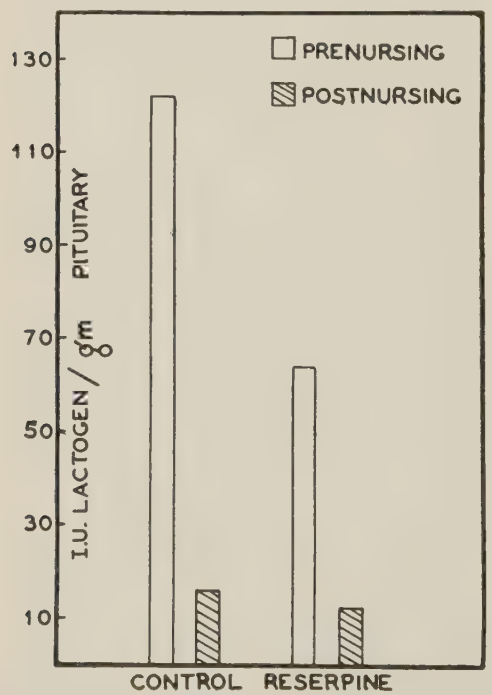

FIG. 1. Effect of reserpine on pituitary lactogenic hormone concentration following 10 hr isolation of mother and young (prenursing level) followed by 30 min. nursing (postnursing level). Reserpine administered 120 min. prior to end of isolation period.

with subsequent inhibition of tropic hormone discharge. Results of the present study indicate that reserpine inhibits oxytocin release centrally rather than peripherally since above normal milk yields were obtained in reserpine-treated animals following injection of oxytocin. These observations would appear to rule out competition between oxytocin and reserpine at the myoepithelial level, at least, to any significant degree. Milk yield greater than normal is probably a result of the dosage of oxytocin employed. Although comparison of responses resulting from hormone administration by different routes of injection is inadvisable, it is of interest that a single intravenous injection of .1 USP/kg oxytocin results in milk yields comparable to those obtained with .2 USP/kg subcutaneously, whereas .05 USP/kg intravenously produced yields comparable to that of controls of the present study(11). Data obtained from animals injected with reserpine 10 minutes prior to litter replacement suggests a certain level of the drug must be present in general circulation before maximum inhibition of milk let-down is assured. It is possible that reserpine may be acting at any of the higher nerve centers involved in the let-down reflex or at centers within the spinal cord. However, it has been shown(12) that reserpine has no effect upon spinal reflex arcs at doses which normally produce symptoms characteristic of reserpine activity. Since ptosis was not evident in rats treated with the drug, it appears that inhibition of milk let-down reflex by reserpine occurs at higher centers. Pharmacological(13, 14) and hormone secretion(6-8) studies indicate the hypothalamus and reticulum as the principal sites of action of reserpine, particularly when low doses of the drug are used.

Recent investigations have shown that atropine and dibenamine inhibit reflex release of both oxytocin and lactogenic hormone(15). These observations suggest that both adrenergic and cholinergic components are involved in the reflex arc. Reserpine has been shown to exert a central sympathetic depressant effect(13). It is probable, therefore, that the drug inhibits oxytocin release through suppression of sympathetic components in the region of the hypothalamus which would result in a reduction in the circulating level of the hormone with subsequent decrease in let-down of milk.

Several investigators(3-5) have suggested reserpine may stimulate production and discharge of lactogenic hormone. The lower prenursing level of animals treated with the drug 120 minutes before end of isolation period appears to substantiate this view. The lower prenursing level of this group may not reflect the actual lactogen releasing activity of reserpine since pituitary restoration occurs at a very rapid rate. Grosvenor and Turner(16) have shown that approximately 50% of the prenursing level is restored within 2-3 hours following 30 minutes nursing. The observation that reserpine may initiate lactogen discharge while at the same time inhibit oxytocin release suggests that the drug may exert a direct action upon the adenohypophysis. On the other hand, the lactogen releasing activity of reserpine may be mediated by suppression of an inhibitory hypothalamic center such as that which has been postulated for control of follicle-stimulating-hormone release(17).

Summary. 1) The effect of reserpine upon release of oxytocin and lactogenic hormone from the hypophysis has been studied in 14-day postpartum lactating rats. Oxytocin discharge, evaluated by milk let-down yield per timed nursing, was significantly inhibited. Results indicate the drug inhibits oxytocin discharge by suppression of central sympathetic centers involved in the let-down reflex. 2) Decrease of pituitary lactogen content (prenursing level) by approximately 48% indicates reserpine stimulates release of lactogenic hormone. It is possible that the effect is mediated by suppression of an inhibitory hypothalamic center controlling lactogen discharge or by direct action of drug upon adenohypophysis.

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Effects of Gastric Juice Fractions on Uptake of Labeled Vitamin B_{12} By Rat Liver Slices. (24930)

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Miller and Hunter(1) have shown that hog intrinsic factor concentrates increase uptake of cobalt^{60} labeled Vit. B_{12} by a rat liver slice. This uptake is concentration dependent with low concentrations increasing uptake and high concentrations inhibiting uptake. Addition of other proteins without known intrinsic factor activity fails to enhance uptake. Recently we found that at all concentrations tested, human gastric juice inhibited uptake of Co^{60} Vit. B_{12} by rat liver slice(2). Herbert found that this inhibitory effect of human gastric juice could be overcome by use of sequential incubation technic(3). He stated that this may be an *in vitro* test of intrinsic factor activity since gastric juice from a pernicious anemia patient failed to show this uptake. Latner, in purification of hog pyloric mucosa was able to separate a fraction that inhibited Vit. B_{12} absorption from a fraction with intrinsic factor activity(4). Using the method of Richmond, we chromatographed individual human gastric contents on IRC resin column(5). We found 3 Co^{60} Vit. B_{12} binding peaks in this effluent. Since the effluent of column of Richmond *et al.* contained intrinsic factor(6,7), we thought it would be of interest to determine if individually these fractions which bind Vit. B_{12} are capable of enhancing uptake of Co^{60} Vit. B_{12} by the rat liver slice and to determine if any of these fractions caused inhibition of Vit. B_{12} uptake produced by human gastric juice.

Technic. Gastric contents were obtained from 2 fasting subjects after stimulation of gastric secretion by 10 units of regular insulin administered intravenously. Patient No. 1 had a duodenal ulcer and 8.7% Co^{60} Vit. B_{12} liver uptake (normal$>$4.7%)(8). Patient No. 2 had no signs of anemia and his gastric contents contained acid and pepsin. He was receiving therapy for chronic urticaria. The fasting gastric contents of these 2 patients were chromatographed from pH 3.1 to 6.1 using 5 buffers pH 3.1, 3.9, 4.5, 5.4 and 6.1 on an IRC-50 cation exchange resin column(5). In this procedure 30 mg of lyophilized, dialyzed and neutralized gastric contents were incubated with 0.3 μc Co^{60} Vit. B_{12} after which they were applied to the column. Radioactivity of the effluent was determined before and after dialysis(2). It is reported on graphs as net counts/minute/ml (ncpm) of effluent over detector background counts/minute. All samples were counted for at least 10,000 net counts. In this analysis (Fig. 1) gastric juice from patient No. 1 produced 5 more or less well defined protein peaks in the effluent. These protein peaks were measured using optical density at 280 mμ. Peak 1, effluent pH 3.1, contains practically all the polysaccharide present in gastric juice. This included 'A' substance. Peak 2A, effluent pH 3.7 to 3.9 and 3, effluent pH 4.2 to 4.4, contain proteolytic activity by the hemoglobin method for measuring peptic activity(9). Peak 2B, effluent pH 3.9, and 4, effluent pH 4.6 to 4.8, contains protein substances of unknown physiologic significance. Since presence of peptic activity may change binding ability and other characteristics of gastric juice, pepsin was inactivated by bringing its pH to 10 for 30 minutes prior to adding Co^{60} Vit. B_{12} to the gastric juice of Patient 2(10). Slices prepared from chilled liver of rats sacrificed by decapitation were immersed in Hastings buffer containing .015 μg of Vit. B_{12} and .015 μc of Co^{60} Vit. B_{12}. Varied amounts by weight of dialyzed, lyophilized human gastric juice fractions were then added. The system was gased with 5% CO_2 and 95% oxygen, incubation was at 37°C for 60 minutes. After incubation the slices were washed with buffer by immersing in fresh buffer. The amount of radioactive B_{12} remaining was measured in scintillation well counter. Uptake of Vit. B_{12} was corrected for weight of tissue and expressed as % difference from con-

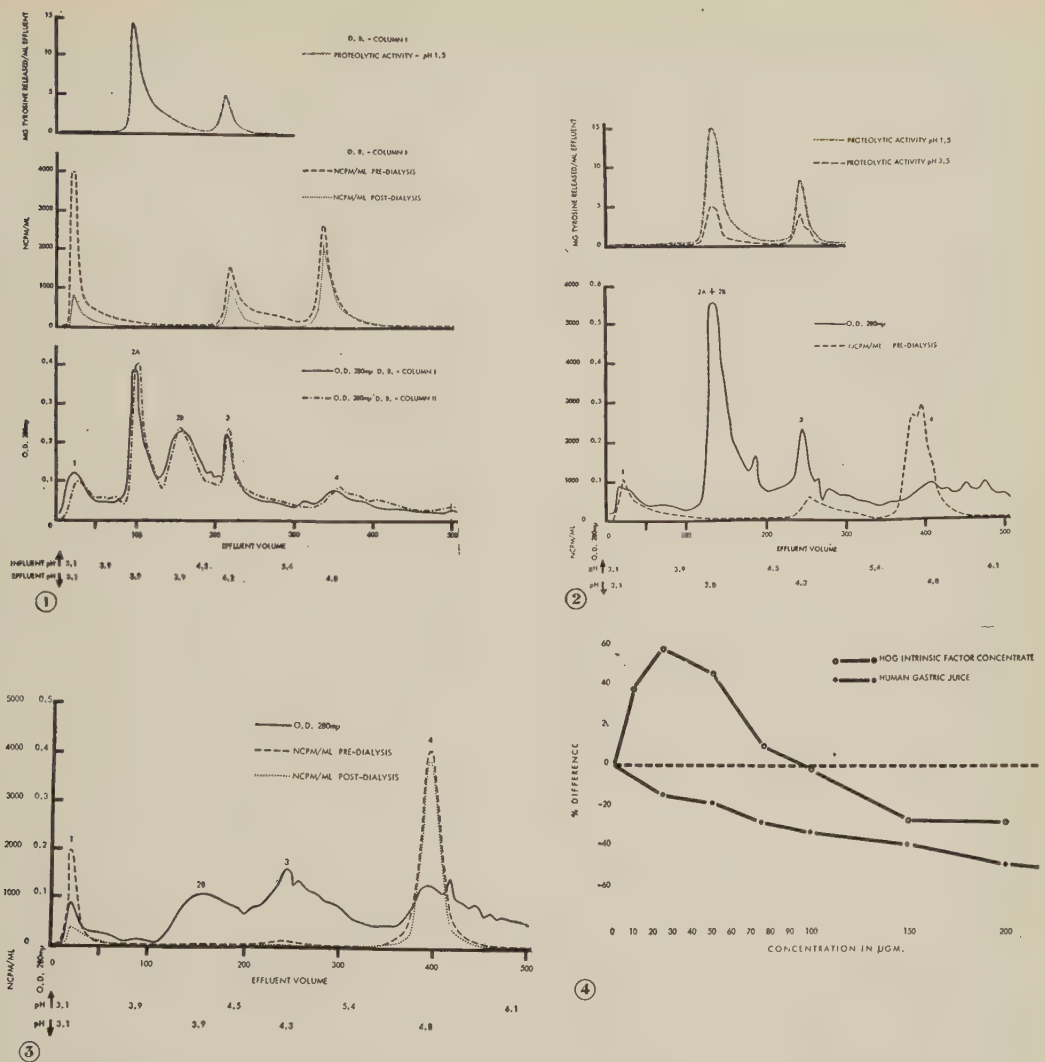

FIG. 1. Chromatographic pattern of Patient 1.
FIG. 2. Chromatographic pattern of Patient 2.
FIG. 3. Chromatographic pattern of Patient 2 after inactivation of proteolytic activity.
FIG. 4. Effect of hog intrinsic factor concentrate and human gastric juice on rat liver slice.

trol value. Uptakes less than the control are indicated by negative numbers. In 35 determinations, mean control uptake was 485 ± 19 (mean ± standard error of mean) net counts/minute/100 mg liver.

Results. Fig. 1 shows resin column separation of gastric contents of Patient 1. The top curve is peptic activity. The middle curve shows that Co^{60} labeled Vit. B_{12} appears in 3 of the 5 protein peaks and that a good portion of radioactivity is nondialyzable and presumably bound to the protein. The bottom 2 curves show the protein pattern obtained by chromatographing gastric contents on 2 separate occasions. Good agreement is noted.

Fig. 2 shows chromatographic separation of gastric contents of Patient 2. Proteolytic activity measured at pH 1.5 and 3.5 and presence of Co^{60} Vit. B_{12} in the effluent are also shown. In the effluent of this column peaks 2A and 2B overlap. The Co^{60} Vit. B_{12} binding peaks of this fractionation were combined prior to dialysis. Percentages of radioactive cobalt incubated with gastric juice and which

TABLE I. Percent Difference from Control Uptake Produced by Protein Peaks of Human Gastric Juice.

Protein peak #	1			2A	2B	3			4		
Patient #	1	2	2 (treated)	1	1	1	2	2 (treated)	1	2	2 (treated)
μg applied											
25	8		48						0		
	19		32						0		
50	25	60	30			0			0		
	33	45	26			0			0		
75						0		26	17		
						0		32	22		
100	19	28	−6	0	3				32		14
	26	31	−5	4	0				35		2
150						24	17	35	55		
						22	21	32	78		
200	−22	−8		0	0	10			28		
	−20	−4		7	0	13			15		
						11					
300						0	55	56			
						0	66	56			
400				16	0					10	41
				11	0					0	27
500		−44		37	28					37	80
		−38		33	20					35	90
600				28	4		29				
					0		15				
700				22	0						
				20	0						
1000										46	
										48	
1500							−45				
							−35				
2500										14	
										3	

appeared in each binding peak were as follows: Peak 1, 14%, Peak 3, 16% and Peak 4, 64%. The remaining 6% was retained by the resin column. These 3 peaks were dialyzed to determine percentage of radioactivity which remained. Of radioactivity found in each peak, 86% remained in Peak 1, 35% in Peak 2 and 100% in Peak 4. Peak 4 is thus by far the strongest binding peak.

Fig. 3 illustrates the effect of inactivating proteolytic activity prior to chromatography. Proteolytic Peak 2A has disappeared while the other proteolytic Peak 3 remains and may have increased slightly. Peaks 1 and 4 remain undisturbed. Of radioactivity incubated with this gastric juice 21% appeared with Peak 1, 2% with Peak 3 and 75% with Peak 4. After dialysis 13% of radioactivity of Peak 1 remained, 12% of Peak 3 and 79% of Peak 4. Again Peak 4 is the strongest binding peak compared both to amount of radioactivity appearing in effluent and ability to make this radioactivity nondialyzable.

Fig. 4 shows the effect of varying concentrations of hog intrinsic factor and gastric contents of Patient 1 on uptake of Vit. B_{12} by the rat liver slice. Small concentrations of hog intrinsic factor* increased uptake of Vit. B_{12} and as the amount was increased, uptake diminished and finally reached a point below control value. Human gastric juice on the other hand showed no enhancement of uptake. Inhibition was present at lowest concentrations, more marked as concentration of gastric juice was increased. This was also true when the entire effluent from column was lyophil-

* W. L. Williams, Am. Cyanamid Co., Pearl River, N. Y.

ized and dialyzed and placed on the liver slice in similar concentrations.

Table I shows results obtained when these dialyzed and lyophilized chromatographic fractions were redissolved and added to rat liver slice. Unlike intact gastric juice, each of these protein peaks was capable of increasing uptake of Co^{60} labeled Vit. B_{12} by the rat liver slice. Like hog intrinsic factor, there is an optimal concentration point for each peak. Destroying proteolytic activity did not seem to affect the results. The peaks that bound Vit. B_{12} were somewhat more active on a μg basis than the other 2 peaks which did not bind Vit. B_{12}.

The areas between protein peaks were tested by rat liver slice technic. Control values were produced in all except the area beyond Peak 4 which showed a slight increase in uptake and inhibition at higher doses. We interpreted this to mean that there was trailing off of activity of Peak 4. Recombination of the effluent of Patient 1 produced only inhibition of the rat liver slice uptake similar to that shown in Fig. 4.

Discussion. We have shown that 5 fractions of human gastric juice are capable of enhancing uptake of Vit. B_{12} by rat liver slice at low concentrations. One fraction contains almost all polysaccharides present in gastric juice and 2 of these fractions contain proteolytic enzyme activity. Three fractions bound Vit. B_{12} as measured by dialysis. This increase in uptake was noted in gastric juice which only produced inhibition of Co^{60} Vit. B_{12} uptake at similar concentrations prior to fractionation and after recombination of the entire effluent. This suggests that each protein peak is capable of producing increased uptake by the rat liver slice when used by itself but, combined with others, inhibition occurs. The cause of this is unknown. That this difference is not due to physical chemical changes produced by the column during chromatography is shown by the fact that recombined effluent acted in a way similar to that of the gastric juice prior to chromatography. One might surmise that these fractions interact with each other producing inhibition when added together. The uptake noted with sequential incubation of human gastric juice could be explained in this way (3), since washing off the gastric juice prior to addition of Vit. B_{12} might leave one of these fractions intact on the liver cells while washing the others from the slice.

Whether enhancement of uptake Co^{60} Vit. B_{12} by the liver slice actually represents an assay for intrinsic factor cannot yet be stated. However, if it is, our result suggests that either several intrinsic factors exist or that intrinsic factor is capable of binding to the protein peaks and in this way it is spread out according to protein concentration in the effluent of the column.

Three of the 5 protein peaks obtained from a chromatography of human gastric juice make Vit. B_{12} nondialyzable. Destroying proteolytic activity prior to incubation with Vit. B_{12} changed the percentage of radioactivity appearing in the second proteolytic peak and decreased slightly the ability of the other 2 binding peaks to make Vit. B_{12} nondialyzable. This is different from the work of Grasbeck who found a decrease in binding and fewer binding peaks after proteolytic inactivation (10). However, his analysis by electrophoresis cannot be directly compared to the fractions obtained by the method used here.

Since we have found that inactivation of the proteolytic activity and presence or absence of Vit. B_{12} binding did not seem to influence materially the effect each fraction had on the liver slice, we could assume that binding of the vitamin to the gastric fraction or the presence of proteolytic activity are not important factors in producing the uptake by the rat liver slice.

Summary. 1. Gastric contents from 2 subjects without pernicious anemia have been chromatographed on an IRC resin column. Five more or less well defined protein peaks appeared in the effluent. Each peak was capable of enhancing uptake of Co^{60} Vit. B_{12} by the rat liver slice. 2. Prior to fractionation and after these gastric fractions have been reconstituted, these gastric juices produced inhibition of Co^{60} Vit. B_{12} uptake by the rat liver slice at all concentrations tested. 3. The effluent of these chromatographic separations of human gastric content produced 3 Co^{60} Vit. B_{12} binding peaks. Destroying proteolytic ac-

tivity of one of these gastric juices decreased the binding of one of these but did not change the results with the liver slice. 4. The enhanced uptake on the rat liver slice produced by these gastric juice fractions did not seem to be related to proteolytic activity.

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Serum Protein-Bound Carbohydrates and Lipids in Cholera. (24931)

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Electrophoretic studies on plasma proteins in cholera showed(1) a diminution in albumin and α-globulin, increase in β-globulin which was further differentiated into β_1 and β_2 fractions and no change in γ-globulin. Plasma proteins are conjugated with carbohydrates and lipids. As distribution of these protein bound hexoses and lipids is likely to be disturbed in pathological conditions(2-8) it was of interest to study glycoproteins, mucoproteins and lipo-proteins in serum of cholera patients. Total lipid, phospholipid and cholesterol of serum were also determined. The above studies were also carried out in normal subjects for comparison.

Methods. Patients admitted into Nilratan Sircar Medical College Hospitals, Calcutta with typical symptoms of cholera were selected. As routine procedure, stool was cultured and cases whose stool showed presence of cholera vibrio were included in the report. Normal subjects were persons admitted in surgical ward of hospital for vaginal hydrocele or hernia. Examinations of blood, urine and stool were carried out to ascertain if subjects were otherwise normal. Blood was collected from anticubital vein in early morning, before breakfast and before any treatment, in centrifuge tubes and clear serum used for different estimations. Glycoprotein and mucoprotein were precipitated from aliquots of serum, dissolved in alkali, treated with orcinol-sulfuric acid and color compared with standard solution of galactose-mannose(9). For estimation of lipo-proteins(10) an aliquot of serum was treated with saturated solution of Sudan Black B in 95% ethanol, alcohol evaporated, centrifuged and supernatant stained serum used for electrophoresis. .04 cc of stained serum was applied to Whatman No. 1 strips, and electrophoretic separation carried out with barbiturate-barbituric acid buffer of pH 8.6 and ionic strength .05 for 8 hours, by using L.K.B. paper electrophoresis apparatus with current strength of 5 mA and 250 volts. Whatman strips were dried at 70°C for half hour, optical density curves of colored zones plotted using Photovolt Photoelectric Densitometer Model 425 on graph paper and areas of component section of graph were measured by planimetry. α,β lipo-proteins and "O" fraction were expressed as percentage of total lipids. Cholesterol was estimated by method of Sobel and Mayer(11), phospholipid by determining P of ether extract of serum(12) and multiplying value by 25(13) and total serum lipids by the method of Bragdon(14). Results are given in Table I.

TABLE I. Serum Protein Bound Carbohydrates and Lipids in Cholera and in Normal Subjects.

	Normal (10)*	Cholera (10)*	"t"
Glycoprotein (mg/100 cc)	194 ± 5 †	272 ± 7	9.2
Mucoprotein (mg/100 cc)	45 ± .7	80 ± 2	16.8
Total lipid (mg/100 cc)	619 ± 3	808 ± 19	9.9
α-lipoprotein (%)	32.7 ± 1.0	21.6 ± 1.0	7.9
β-lipoprotein (%)	59.3 ± 1.0	65.7 ± 1.0	4.6
"O" fraction (%)	8.0 ± .7	12.6 ± 1.0	3.8
Phospholipid (mg/100 cc)	250 ± 6	389 ± 16	8.1
Cholesterol (mg/100 cc)	160 ± 3	137 ± 2	6.4

* No. of subjects. † Mean ± stand. error.

Results. In cholera serum phospholipid, total serum lipid as well as β-lipoprotein were significantly increased with decrease in α-lipoprotein and total cholesterol. Serum glycoprotein and mucoprotein fractions also increased significantly as seen in Table I.

Discussion. β-globulins are increased while α-globulins are diminished(1). The increases in cholera of β-lipoproteins and decrease in α-lipoproteins are commensurate with changes in α and β-globulins observed and are similar to changes reported in the nephrotic syndrome (15,16). Phospholipids and total lipids are greatly increased in cholera. This may be associated with mobilization of fat from depots, or to liver damage. The α-protein elevations may be associated with inflammation and tissue destruction(2-5).

Summary. Serum glycoproteins, mucoproteins, phospholipid, total lipid, total cholesterol and lipoproteins were determined in patients suffering from cholera and in normal subjects. In cholera serum glycoproteins, mucoproteins, phospholipids, total lipids and β-lipoproteins were significantly increased and α-lipoproteins and total cholesterol were diminished.

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Effect of Coprophagy on Utilization of Urea by the Rat. (24932)

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Our recent studies as well as elsewhere(1) have demonstrated a growth stimulatory effect of urea, in rats, when added to diet containing minimum level of essential amino acids but lacking in non-essential amino acids. This growth response was attributed to extra nitrogen supplied by urea used for synthesis of non-essential amino acids. The way in which *in vivo* synthesis of non-essential amino acids from urea proceeds is not clear but it is logical to assume that the first step is splitting of urea by urease. Presence of this enzyme was demonstrated in gastrointestinal tract of ruminants as well as many simple-stomached animals, including cats, dogs, mice, rats and guinea pigs. The source of urease in monogastric animals was debatable for some time but it is apparent from latest experiments, reviewed by Kornberg and Davies(2), that *in vivo* breakdown of urea is due ultimately to the presence of urea-splitting organisms in the intestinal tract rather than to gastric urease *per se*. The beneficial effect of intestinal bacteria by splitting down urea and thus making available to the host extra nitrogen for synthesis of amino acids is comparable with bacterial synthesis of vitamins in the intestinal tract. The most recent studies pertaining to the latter problem(3,4) have demonstrated that availability to the host of certain vitamins that are produced by intestinal synthesis is considerably reduced if not completely abolished, when the animals are prevented from eating their feces (coprophagy). Our purpose was to find out whether prevention of coprophagy in the rat would have a similar depressing effect on utilization of urea. The use of urea in nutrition studies with man(5) and the fact that man, in general, does not practice coprophagy makes this question one of fundamental as well as practical importance.

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Methods. Twenty-four male weanling albino rats (purchased from Holtzman) were divided into 4 groups consisting of 6 animals each. Each rat was housed in individual wire screen-bottom cage in room at 72°C and was permitted to eat and drink *ad lib.* The first 2 groups of rats were given a synthetic diet containing essential amino acids at their minimal required levels while the other 2 groups were fed a comparable ration supplemented with urea. Composition of basal diet and the amino acid mixture has been described previously(6). In one group of rats on each diet, coprophagy was prevented. This was accomplished by placing a plastic feces collection cup over the anus and tail of the rat, as described by Barnes *et al.*(7). Emptying of feces from the cups and weighing of animals was done every other day during 19-day experimental period. Then the animals were bled and the blood saved for cholesterol analyses.

Results. Results are presented in Table I. Rats which ingested a complete diet containing urea as source of "non-essential" nitrogen, gained an average of 52 g while those fed a ration devoid of urea gained only 37 g during 19-day experimental period. When coprophagy was prevented in animals on the former diet there was no change in growth response to urea, as is evident from body gain of 51 g. Similarly treated animals, which were fed a regimen devoid of urea, gained 32 g which is somewhat lower but probably not significantly different from the gain of control animals which had access to their feces.

Rats fed urea-supplemented diet consumed more food and utilized it more efficiently than did animals on a urea-free diet. Coprophagy did not seem to affect either consumption or utilization of the diets.

Serum cholesterol values (Table I) followed, in general, the weight gains of the animals. Since body weight was invariably related to amount of food consumed it is pos-

TABLE I. Average Weight Gain, Food Consumption, Food Efficiency and Blood Serum Cholesterol in Conventional and Coprophagy-Prevented Rats, Fed Urea-Free or Urea-Supplemented Diets.*

Diet	Coprophagy	Wt gain (g)	Food intake (g)	Food efficiency	Serum cholesterol (mg/100 ml)
Basal	+	37 ± 2†	152	.24	104
	—	32 ± 1	142	.23	93
Basal + urea	+	52 ± 3	178	.29	113
	—	51 ± 1	174	.30	113

* Six rats with avg initial wt of 52 g were used in each exp. † Stand. error of mean.

sible that the latter also affected cholesterol values.

Discussion. Our experiments confirm previous observations(1) of growth promoting effect of urea when fed with the diet which was deficient in non-essential amino acids. The present study further shows that prevention of coprophagy apparently does not reduce availability of urea to the animals. This finding is contrary to previous observations on the effect of coprophagy on availability of intestinally synthesized vitamins(3,4). The probable explanation of this difference lies in the animals' ability or inability to absorb nutrients from the intestinal tract. Intestinal synthesis of vitamins occurs, for the most part, in the large intestine where their absorption is apparently low.

Effective utilization of urea by animals that had no access to their feces suggests the possibility that following enzymatic splitting of urea by intestinal microorganisms, the released ammonia is immediately absorbed by the intestine rather than incorporated into the microbial protein. The ammonia is then transported *via* blood to the liver where it can be used for biosynthesis of non-essential amino acids or reconverted back to urea. This interpretation is in accord with the demonstration of bacterial urease throughout the gastrointestinal tract(2), including the stomach, and the rapid absorption of ammonia from the cecum and other parts of the gastrointestinal canal(8).

Summary. 1. Addition of urea to a synthetic amino acid diet lacking in non-essential amino acids stimulated growth of young rats. 2. The growth-stimulatory effect of urea was not affected by prevention of coprophagy in the animals.

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Effects of Inanition, Protein Depletion and Repletion on Serum Lactic Acid Dehydrogenase Levels in Rats.* (24933)

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Since the initial report by Hill and Levi(1) on elevation of serum lactic acid dehydrogenase levels (SLD) in neoplastic disease, numerous clinical and experimental studies concerned with changes in SLD concentrations in a wide variety of pathologic states have been reported. In addition to cancer, increased enzyme levels have been observed in myocardial infarction, leukemia, anemia, diabetic acidosis, obstructive jaundice, infectious mononucleosis, hepatitis, muscular dystrophy, and other unrelated pathologic states(2,3). Inasmuch as cachexia, anorexia, and reduced food intake may be associated with above conditions, it was of interest to investigate the effects of level of dietary protein upon values of SLD. In the present study, the effects of inanition, protein depletion, and of repletion with diets containing 5 different levels of protein, on serum concentrations of SLD and total protein and on hematocrit values of blood have been investigated.

Materials and methods. Animals. Adult, male Sprague-Dawley rats were housed singly in suspended type wire cages with wire bottoms. They were maintained on Purina Laboratory Chow and tap water until initiation of the study. *Inanition.* Food was withheld from rats until they lost 25% of their original weight, the depletion requiring 8 days. Drinking water was available at all times. *Protein-free.*† Animals were fed the protein-free diet previously described(4) until they lost 25% of weight, the depletion requiring approximately 22 days. *Repletion.*† The 8, 17, 40 and 64% protein diets contained 10% vegetable oil, 4% salt mixture, (U.S.P. XIV), and the vitamin diet fortification mixture incorporated into the protein-free diet(4). The contents of casein and starch were adjusted to yield the desired protein level. For the 25% level of protein, ground Purina Laboratory Chow was employed(5). Rats were fed protein-free diet until they had lost 25% of weight. They were then repleted with diets containing the various levels of protein. When they had regained their original weight, the animals were bled. All diets were fed *ad lib. Chemical and hematologic analyses.* To avoid hemolysis, blood samples were obtained from the tail and centrifuged immediately for SLD determinations. Following this procedure, the animals were bled by cardiac puncture under light ether anesthesia. SLD activity was determined by the method of Hill and Levi(1), employing a Beckman Model DU spectrophotometer. Total serum protein and hematocrit determination were carried out by procedures previously reported(6). Analysis of data was made by standard statistical procedures employing the *t* test(7).

Results. The influence of dietary regimens on serum lactic acid dehydrogenase activity and other values is summarized in Table I. The results of hematocrit determinations indicated the development of hemoconcentration concomitant with depletion followed by a hemodilution during repletion. The effects were most pronounced in the starved group. The observations are in accord with the studies reviewed by Allison(8) which demonstrated that alterations in plasma volume may accompany depletion and repletion.

The general effects of fluctuations in plasma volume were to enhance the magnitude of the SLD changes and to minimize those of the total protein. Following a 25% weight loss produced by either starvation or protein depletion, significant elevations occurred in SLD concentrations. When the changes in optical density were corrected for increased hema-

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† Obtained from Nutritional Biochemicals Corp., Cleveland.

TABLE I. Effects of Inanition, Protein Depletion and Repletion on Serum Lactic Acid Dehydrogenase Levels.

Group	No. rats	Initial wt (g)	Depleted wt (g)	Repleted wt (g)	Hematocrit (%)	Total serum protein (g%)	SLD (ΔO.D. $\times 10^3$)
Normal	26	407 ± 4.4			45 ± .4	5.8 ± .06	17 ± 2.5
Inanition	22	404 ± 6.1	306 ± 4.6*		53 ± .6*	5.2 ± .08*	39 ± 3.2*
Protein-free diet	19	403 ± 6.6	304 ± 5.2*		47 ± .5*	5.0 ± .06*	28 ± 4.6*
8% protein	16	403 ± 5.8	306 ± 4.5*	400 ± 6.3	43 ± .5*	5.5 ± .07*	36 ± 6.8*
17% "	18	399 ± 5.8	304 ± 4.1*	401 ± 5.6	43 ± .8*	6.5 ± .09*	10 ± 2.4*
25% "	12	407 ± 3.3	308 ± 2.8*	408 ± 3.6	41 ± .6*	6.1 ± .06*	12 ± 2.1*
40% "	15	405 ± 3.6	303 ± 3.3*	400 ± 3.9	40 ± .4*	6.2 ± .08*	13 ± 1.2*
64% "	17	406 ± 2.6	309 ± 2.2*	406 ± 3.3	43 ± .5*	6.1 ± .07*	10 ± 1.8*

Findings include stand. error of mean.
Statistically significant differences from normal values are indicated: * P = <.01.

tocrit by the formula:

$$\frac{(100 - H_2)\ (\text{observed } \Delta \text{ O.D.})}{(100 - H_1)}$$

in which H_1 = normal hematocrit and H_2 = experimental hematocrit(9), the differences were still highly significant. Upon repletion, SLD levels declined to less than normal in all but the 8% protein group. The increased SLD activity of this group was consistent with the decreased serum protein concentration but the cause was not apparent. SLD levels in some patients with cancer have also declined following administration of large amounts of protein(10).

The major factor contributing to the increased SLD levels was apparently degradation of somatic tissue, with release of the enzyme into the circulation. It is well established that skeletal muscle which quantitatively undergoes the greatest loss in weight during depletion(11) contains large amounts of lactic acid dehydrogenase(12). This interpretation is in accord with the clinical observations of White(3) who determined SLD and creatine excretion.

Subnormal concentrations of SLD following weight repletion on an adequate protein diet may be due to several factors: (a) in part to hemodilution (b) tissue protein may compete with SLD for common precursors (c) preferential synthesis of tissue protein during repletion (d) increased anabolism during repletion may increase tissues demand for SLD, resulting in release of reduced amounts to serum. The significant increases in total protein concentrations after repletion provide evidence that the mechanisms for protein synthesis were not injured during the depletion regimens. Increased concentrations of SLD in pathologic states, characterized by inflammation or abnormally rapid cellular growth, suggest that mechanisms other than tissue degradation may also play a role in increased SLD concentrations(2).

Summary. The effects of inanition, protein depletion and repletion upon serum lactic acid dehydrogenase (SLD), total serum protein and hematocrit values have been determined in adult, male Sprague-Dawley rats. Significant increases in concentration of SLD concomitant with decreases in serum protein levels followed inanition and protein depletion. Upon repletion, subnormal SLD values occurred in groups fed a diet containing 17% or more of protein. Serum protein values for the groups were significantly increased. The results are discussed with respect to possible causes for observed changes.

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Rapid Reversal of Ethionine Inhibition of Enzyme Induction in *Pseudomonas aeruginosa* by L- and D-methionine. (24934)

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A number of amino acid analogues are incorporated into bacterial protein when the cells are grown in their presence (*cf.* 1 for review of literature). Presumably this also occurs when enzymes are induced in resting cell suspensions although proof is lacking. Enzyme induction is inhibited by amino acid analogues in such cells and the appropriate amino acid when added with the analogue prevents the inhibition. It was of interest to determine how rapidly an amino acid can reverse the analogue inhibition once it is established. This would give an indication of the rate the analogue can be displaced from the site or sites to which it is attached.

Methods. A strain of *Pseudomonas aeruginosa* was grown for 24 hours at 35° in 100 ml Difco nutrient broth. The cells were centrifuged, washed in water and centrifuged again. They were then suspended in 12 ml of 0.05 M Na-K-phosphate pH 7.6, and 0.5 ml of this suspension was used in each Warburg vessel which had a final fluid volume of 2 ml. Oxidation of 0.5 mg Na benzoate was measured. This proceeds by simultaneous induction of several enzymes(2). Because of the number of enzymes involved and the complexities of oxidative reactions no conclusion can be drawn about the locus of action of the analogue. But oxygen uptake measurements can accurately detect changes in enzyme activity and the rate at which they occur.

Results. Fig. 1 shows results with DL-ethionine. It was added with the benzoate at zero time and after 90 minutes produced a constant 63% inhibition of oxygen uptake. Oxidation of benzoate alone began after a 30 minute latent period and reached a linear rate after 90 minutes. After 120 minutes L- or D-methionine was added. When added to the vessels containing only benzoate the 2 isomers had no effect on the rate. Ethionine added at this time was also without effect. When the 2 isomers were added to the vessels in which ethionine had been present from the beginning rapid reversal occurred. For the L-isomer this became evident in 15 minutes, almost complete during the next 15 minutes. For the D-isomer, reversal was not apparent in the first 15 minutes but occurred rapidly during the next 15 minute period. The initial difference between the 2 isomers was small but consistent. Exactly the same results were obtained with 1.0 μg p-fluorophenylalanine and 0.2 μg of L- or D-phenylalanine. Phenylpyruvic acid is also an antagonist for this analogue and it behaved initially like the D-isomer. Addition of amino acids other than the specific ones failed to reverse the inhibitions.

Three other facts may be briefly mentioned. If the methionines were added 60 minutes before ethionine and benzoate no reversal was evident presumably because both the L- and D-isomers were metabolized. On the other hand, if the amino acids were incubated 60 minutes with ethionine before the addition of benzoate reversal occurred presumably because ethionine had inhibited the metabolism of both isomers. Finally, if the nitrogen pool was increased by a prior incubation of the cells with 30 μg $(NH_4)_2SO_4$ and 4.3 μg succinic acid, ethionine inhibited to the same extent as in the untreated cells. Similar results were obtained with p-fluorophenylalanine.

Discussion. No conclusions can be drawn from these experiments about the site of action of ethionine but several possibilities may

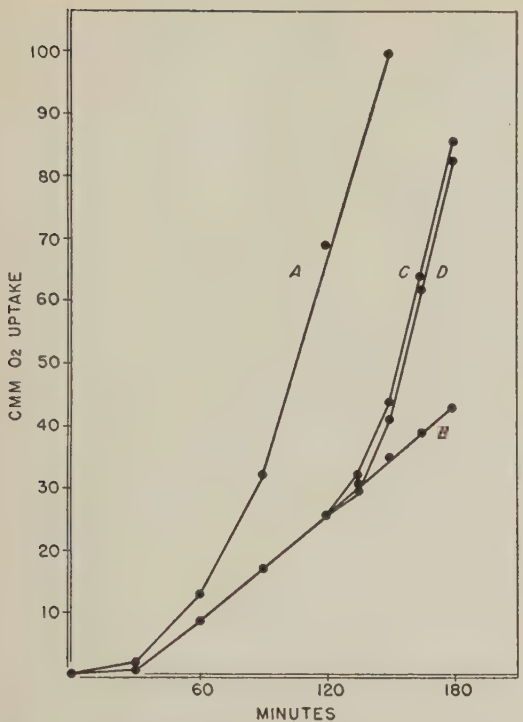

FIG. 1. Effect of 20 μg DL-ethionine and 2 μg of either L- or D-methionine on oxidation of 0.5 mg Na benzoate. Curve A: oxidation of benzoate. At 120 min. either L- or D-methionine or ethionine was added. There was no effect on oxidation rate. Curve B: oxidation of benzoate in presence of ethionine added at zero time. At 120 min. L-methionine (curve C) or D-methionine (curve D) was added.

be mentioned. After an initial period ethionine caused a constant inhibition. If it had merely delayed the entry of benzoate into the cells, *i.e.*, if oxidation rate was limited by amount of substrate available the inhibition should have decreased with time, because benzoate would be entering the cell throughout the entire experimental period. Since the inhibition remained constant, it seems probable that ethionine was not inhibiting a permease. Secondly, if upon addition of methionine the cell synthesized enzyme *de novo* and thus bypassed the ethionine inhibition, any amino acid should have been effective. The specificity of the reaction indicates that ethionine was displaced. Thirdly, if ethionine was incorporated into the enzyme protein during the 120 minute period, then methionine must have rapidly displaced it to convert an inactive into an active enzyme. Fourthly, the slightly slower action of the D-isomer may mean either that it was assimilated more slowly or that it was changed to the L-isomer by an isomerase.

Summary. 1) Ethionine inhibition of induction of enzymes involved in benzoate oxidation by washed cell suspensions of *Pseudomonas aeruginosa* is rapidly reversed by L-methionine and almost as rapidly by D-methionine. Neither amino acids nor ethionine have any effect once the enzymes have been induced. Similar results were obtained with p-fluorophenylalanine.

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Stimulation of Mitosis in Rat Marrow Cultures by Serum From Infected Rats.* (24935)

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Workers have reported substances in blood, urine, or inflammatory exudates which are capable of increasing the number of circulating leukocytes(1,2). However, the specific mechanism by which this effect is produced has not been determined. It has not been demonstrated, for instance, whether the observed leukocytosis results from increased mitotic activity in hematopoietic centers or from increased release of mature cells from these centers or other extravascular storage sites.

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In the present experiments, an attempt has been made to demonstrate at least one of the mechanisms by which granulocytosis, which normally accompanies acute infection, is produced. The effect of serum from infected rats on mitotic index of rat marrow cells in short-term tissue culture has been measured; and the results suggest that, in acute infections, some serum-borne substance increases mitotic activity of bone marrow cells and thus, presumably, increases production of mature granulocytes.

Materials and methods. The tissue culture method used was a slight modification of Osgood's "gradient method"(3). Rat bone marrow was expressed from femurs of young, adult male Webster rats with compressed air and immediately suspended in cold tissue culture medium (TC-199, Difco) with vigorous agitation. The cells were counted and then promptly planted in culture medium consisting of TC-199 and 10% rat serum, with penicillin and streptomycin added. Serum and marrow cells were generally obtained from the same rat. Cultures were planted in small screw-top vials (60 x 25 mm) with initial culture volume of 15 ml and cell concentration of approximately 3×10^6 cells/ml. Ordinarily, enough marrow cells were obtained from one rat for 3 such cultures. A glass slide was placed in each culture bottle at an angle of 60°, and marrow cells settled out and grew on the slide and on bottom of bottle. Cultures were incubated over night at 37°C without agitation. The slide was then removed, stained, and examined for evidence of mitotic activity. In almost all instances, occasional mitoses were observed among marrow cells on the slide. Very rarely, cultures failed to show any mitotic activity and so were discarded at this stage. Otherwise, healthy marrow cells were harvested from culture bottles by discarding approximately ⅔ of the supernatant medium, and then by vigorously agitating the remainder with sterile pipette to loosen cells adhering to bottom. The resultant suspension of cultured marrow cells was used to set up the actual test cultures. Test cultures were similar to primary cultures just described except that, for convenience, volumes of 8-10 ml and cell concentrations of 1.5 to 2.0×10^6 cells/ml were used. The composition of test cultures was approximately one-third cell suspension, one-third fresh TC-199, and one-third serum from acutely infected rats or from uninfected control rats. Acute infections were produced by intrathoracic injection of one ml of 24-hour broth culture of *Staphylococcus aureus*. Rats thus injected usually developed, by the following day, an inflammatory reaction in the pleural cavity, characterized by large amounts of cloudy exudate and numerous fibrinopurulent pleural adhesions, as well as marked granulocytosis in peripheral blood. Some variability in response was observed: occasional rats died in less than 24 hours, while others developed little or no inflammatory response. Only rats showing a good exudative reaction and obvious peripheral granulocytosis were included in the study. Serum was obtained from infected rats, as well as from uninfected control animals, by cardiac puncture. Control animals were of same age and weight as experimental rats, and only animals with initial total white blood cell count under 15,000 (less than 30% granulocytes) were used. Preliminary studies had shown that rats with initial counts above these arbitrary levels usually had evidence of infection at autopsy and their sera gave variable results. A few test cultures were set up with serum component replaced by inflammatory exudate, either undiluted or diluted with control serum. Test cultures were incubated at 37°C; and 2 to 3 hours later colchicine, in final concentration of 1×10^{-7} M, was added to arrest, in metaphase, all subsequent mitoses. After additional 17 to 19 hours incubation, the cultures were terminated and the harvested cells washed, treated with hypotonic salt solution, then fixed and stained with acetic-orcein(4). The resultant suspension of stained cells was spread on slides and lightly squashed under a cover slip. Processing the cells in this fashion caused enough spreading of chromosomes to make identification of cells in mitosis extremely easy. Mitotic indices were determined by counting at least 2000 cells from each culture. Differential counts were also made on cell suspensions, distinguishing only between mature granulocytes and all other cells. By this stage, very

TABLE I. Effect of Serum from Infected Rats on Mitosis in Rat Marrow Cultures.

Exp. No.	Mitotic index (No. mitoses/1000 cells) Cultures with serum from infected rats*	Cultures with control serum
1	35	10
2	31	13
3	40	20
	33	
4	11	4
	4	
5	27	13
	24	
Mean†	26	12

* Each figure represents a culture containing serum from different infected rat.
† For difference of means, $P < .05$.

few cells of the erythroid series remained in the cultures.

Results. Table I gives results obtained in 5 experiments in which mitotic activity of 8 marrow cultures containing serum from 8 different infected rats was compared with that of control cultures. All cultures in any one experiment were set up with marrow cells from the same rat. In 7 of the 8 cases, serum from infected rats had a pronounced stimulatory effect on marrow cells, generally resulting in mitotic indices 2 to 3 times that of control cultures. With exception of Exp. 4, mitotic indices in control cultures were quite uniform, ranging from 10 to 20. That both experimental and control values were unusually low in Exp. 4 suggests that viability of marrow cells in this experiment was poor; and examination of slides from the primary cultures of this marrow revealed an exceptionally low number of mitoses.

In a few instances, test cultures were set up containing serum from rats in which the injected staphylococci had failed to "take" and hence a good exudative response had not developed. In these cases, no stimulatory effect of serum was demonstrated; mitotic indices of these test cultures were no greater than control values. Similarly, in studies in which test cultures contained the *inflammatory exudate* instead of serum from infected animals, no stimulatory effect was demonstrated. Undiluted exudate, which undoubtedly contained many viable bacteria, was definitely toxic to marrow cultures; and, even when the exudate was diluted 10-fold with normal serum, mitotic indices in treated cultures were either the same or less than in control cultures.

Differential counts done on harvested marrow cells revealed, in all experiments, essentially the same percentage of mature granulocytes in cultures treated with serum from infected rats as in control cultures. The percentage of mature granulocytes varied only slightly (60% to 75%) from experiment to experiment. It had been hoped that differential counts would indicate whether serum from infected rats increased maturation rate of myeloid cells, since with a relatively constant number of cells in cultures during test period, a higher percentage of mature granulocytes, as compared to controls, would have indicated a more rapid rate of maturation. Unfortunately, in *control* cultures, two-thirds to three-fourths of the harvested cells were mature granulocytes, so that conditions were obviously not suitable for quantitatively demonstrating presence or absence of a maturation-stimulating factor in the experimental cultures. Granulocytes in *all* cultures were maturing at such a rapid rate that any additional maturing effect of serum in experimental cultures would not have been apparent.

Discussion. The results indicate that serum of rats with an acute infection is capable of producing a 2- to 3-fold increase in mitotic rate of rat marrow cells *in vitro*. This finding suggests that peripheral granulocytosis normally accompanying acute infection is due, at least in part, to direct mitogenic effect of some serum-borne substance on myeloid cells in the marrow. It is obvious, of course, that other mechanisms must also be involved. The peripheral granulocytosis during the first 48 hours of acute infection could hardly be due to increased mitotic activity of myeloid precursors since granulocytes ordinarily mature for 2-3 days in the marrow after their last division before being released into peripheral blood(5).

Time and magnitude of observed mitogenic effect is comparable to what Swann, in his recent review of mechanisms controlling cell di-

vision(6), calls a "short latent period" response, the prompt 2- to 3-fold increase in mitotic rate typically seen in tissues with normally high mitotic index upon exposure to various stimuli which speed up energy metabolism.

No attempt has been made, as yet, to identify the agent in serum responsible for its demonstrated marrow-stimulating activity. Perhaps an adrenal hormone, released as part of the body's response to "stress" of acute infection, constitutes the circulating marrow stimulant. It has been demonstrated, for instance, that cortisone may have a myelopoietic effect on bone marrow(7).

However, most earlier studies have indicated that substances responsible for leukocytosis accompanying acute inflammation are produced at the inflammatory site itself(1,8). In our study, this substance might be a product of either the infecting bacteria, disintegrated leukocytes, or the inflamed tissues. Failure to demonstrate a direct marrow-stimulating activity of the exudate itself does not rule out any of these possibilities. The contaminated purulent exudates in our experiments might contain a high enough concentration of substances toxic to marrow cultures to obscure the effect of any marrow-stimulating factor; or it is possible that, *in vivo,* such a locally-produced substance might act on marrow only *indirectly,* through the endocrine system. Such an indirect action of the exudate would, of course, not be demonstrable in our tissue culture system.

Summary. Serum from acutely infected rats produced a 2- to 3-fold increase in mitotic index of rat marrow cells in short-term tissue cultures. These results suggest that a serum-borne marrow-stimulating substance may play a part in producing the characteristic peripheral granulocytosis which normally accompanies acute infection.

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Histidine Metabolic Loading Test to Distinguish Folic Acid Deficiency From Vit. B_{12} in Megaloblastic Anemias.*† (24936)

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In the past, differentiation of clinical deficiency of folic acid from that of Vit. B_{12} in megaloblastic anemias has depended upon extended therapeutic trials with Vit. B_{12} and folic acid. Failure of hematologic response to B_{12}, but a typical reticulocyte and red blood cell rise following minute doses of folic acid indicated folic acid deficiency(1). Formiminoglutamic acid (FIGLU) has been shown to accumulate in urine of patients with folic deficiency but not in uncomplicated Addisonian pernicious anemia or normal subjects

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(2,3). Because many patients with megaloblastic anemia and suspected folic acid deficiency did not excrete significant quantities of urinary FIGLU, it was reasoned that differences in FIGLU excretion by folic acid deficient individuals may vary with dietary intake of protein containing histidine, the amino acid from which FIGLU normally arises. A histidine metabolic loading procedure was thus devised to equalize the quantity of histidine presented for metabolism in all individuals. This uniformly permitted differentiation of clinical folic acid deficiency from that of Vit. B_{12}. Details of this test are reported herein.

Materials and methods. Patients with various types of megaloblastic anemia were studied. Diagnosis of megaloblastic anemia was established in each case by bone marrow megaloblastic erythroid and myeloid pattern, peripheral blood macrocytic anemia, hypersegmented macropolymorphonuclear leucocytes and, often, thrombocytopenia. In non-pernicious megaloblastic anemia, gastric acidity was usually normal, and evidence of combined system (CNS) disease was always absent. Serum Vit. B_{12} level and gastrointestinal absorption of radioactive Vit. B_{12} was normal except in macrocytic anemia associated with sprue or malabsorption syndromes, in which they were decreased. Megaloblastic anemia of Addisonian pernicious anemia type in relapse was characterized by histamine refractory gastric achlorhydria, low serum Vit. B_{12} level and markedly decreased gastrointestinal absorption of radioactive Vit. B_{12}, which improved following administration of Vit. B_{12} with intrinsic factor concentrate. Whenever possible, patients with non-pernicious megaloblastic anemia were given therapeutic trials with 10-25 γ parenteral Vit. B_{12} followed by 1-1.5 mg parenteral folic acid. They invariably made a typical response to folic acid but not to Vit. B_{12}(1). Pernicious anemia in relapse did not respond to 1-1.5 mg folic acid, but responded dramatically to 10 γ parenteral Vit. B_{12}. Control subjects included children of different ages, adults and pregnant women at various stages of gestation. A metabolic load of L-histidine monohydrochloride, 15 g daily, was given in 3 divided doses one-half hour before meals in apple juice or water for 2 to 3 days. Urine was collected under acid as previously described(3). Urinary FIGLU was measured by modification of enzyme technic of Tabor & Wyngarden(4).

Results. After 48-72 hours on this metabolic loading procedure, patients with non-pernicious megaloblastic anemias listed in Table I had urinary FIGLU excretion rang-

TABLE I. Urinary FIGLU Excretion following 48-72 Hr Histidine Metabolic Loading.

	No. of patients	Urinary FIGLU γ/ml	Urinary FIGLU mg/24 hr
Non-pernicious megaloblastic anemia:			
Nutritional macrocytic anemia	12	125– 750	250– 550
Macrocytic anemia of pregnancy	14	90– 875	225– 600
Macrocytic anemia associated with sprue or malabsorption syndromes	10	100– 920	185– 675
Megaloblastic anemia of infancy	3	125–1909	625–2047
Macrocytic anemia associated with liver disease	6	300–1052	300–1850
Addisonian pernicious anemia in relapse:	18	2–30	1.5–35
Non-megaloblastic anemia:			
Iron deficiency anemia	7	1.0–20	1.7–24
" " " of pregnancy	9	1.2–23	1.9–28
Acute leukemia	8	.5–15	.3–16
Chronic "	2	.3, 20	.6, 25
Aplastic anemia	2	.1, 7.1	.9, 8.2
Hodgkins disease	1	13.4	13.3
Chronic hemolytic anemia	3	3.1– 7.2	5.5–11
Anemia associated with gastrointestinal malignancy	6	1.1–28	1.5–30
Control subjects:			
Normal children	8	.1–10	1.9– 7.2
" adults	25	.1–28	.5–30
" pregnant women	10	.5–25	1.2–26

ing from 90 to 1909 γ/ml; final 24-hour output varied from 185 to 2047 mg. In sharp contrast, patients with uncomplicated Addisonian pernicious anemia in relapse, following identical metabolic loading, excreted only 2-30 γ FIGLU/ml urine; final 24-hour urinary FIGLU output ranged from 1.5 to 35 mg. In fact, most such patients rarely achieved FIGLU urinary concentration at 72 hours of 18 γ/ml and 24-hour excretion rarely over 15 mg, values well within the normal range.

Patients with non-megaloblastic anemias and other disorders listed in Table I, when subjected to histidine metabolic loading procedure outlined, showed urinary FIGLU concentration ranging from 0.1 to 28 γ/ml. The final 24-hour output varied from 0.6 to 30 mg. Control subjects, as well as pregnant women at various stages of gestation without apparent disease or hematologic abnormality, after the histidine metabolic loading described, had urinary FIGLU concentration ranging from 0.1 to 28 γ/ml and 24-hour output ranging from 0.5 to 30 mg. Most normal subjects had urinary FIGLU concentration of 0.3 to 5 γ/ml; a 24-hour output of 1-4 mg.

Discussion. It is apparent from our data that individuals with non-pernicious megaloblastic anemia due to folic acid deficiency, as judged from hematologic response to minute doses of folic acid, but refractory to parenteral Vit. B_{12}, excrete from 3 to over 1000-fold the quantity of FIGLU excreted by patients with Addisonian pernicious anemia, in which deficiency is that of Vit. B_{12}. Subjects with other anemias and disease in which no therapeutic benefit to folic acid could be demonstrated, and hence in whom no folic acid deficiency existed, also excreted decidedly normal amounts of FIGLU after this standardized histidine metabolic load.

Increased urinary FIGLU excretion after histidine loading, as described herein, reflects biochemical insufficiency of folic acid cofactors(5,6,7) in such patients. The test thus offers a biochemical index for diagnosis and assessment of clinical folic acid deficiency and a means of differentiating megaloblastic anemias due to folic acid deficiency from those due to Vit. B_{12} deficiency, a problem of considerable clinical importance for which no direct method of this short duration was previously available.

The 15 g histidine monohydrochloride metabolic load was arrived at after many trials with various other dosages. At this level, no patient with uncomplicated pernicious anemia in relapse tested to date showed above normal excretion. All patients with other types of megaloblastic anemia tested showed an excretion value sufficiently above normal to be unequivocal. In fact, certain individuals with minimal megaloblastic changes, in which folic acid deficiency could at best be classified as "borderline" or "subclinical" were distinctly diagnosable. Normal individuals as well as those with megaloblastic anemia due to uncomplicated pernicious anemia could not be induced to excrete a urinary concentration of FIGLU above 30 γ/ml until loading doses of 36 to 45 g per day were administered. Salts of histidine or an equivalent amount of free base are equally effective.

Summary. 1) A new procedure for diagnosis of clinical folic acid deficiency is described which distinguishes it from Vit. B_{12} deficiency. This permits differentiation of Addisonian pernicious anemia in relapse from megaloblastic anemia due to folic acid deficiency. 2) The procedure is based upon oral administration of 15 g of L-histidine monohydrochloride for 48-72 hours and measurement of urinary concentration or 24-hour excretion of formiminoglutamic acid, which accumulates in folic acid deficiency. Under these conditions, urine FIGLU levels do not exceed 30 γ/ml in non-folic acid deficient individuals; are 3 to 1000 fold greater in folic acid deficiency.

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Wound Healing: Investigation of Proteins, Glycoproteins, and Lipids of Experimental Wound Fluid in the Dog.*† (24937)

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Wound fluid provides an external environment for proliferating fibroblasts and remains in contact with repair tissue of experimentally induced wounds for prolonged periods of time. Due to technical difficulties in obtaining this fluid, little is known of its biochemical composition and alteration during time-course of healing. A method of obtaining wound fluid was developed by Schilling *et al.*(2,3). Stainless steel mesh cylinders were implanted subcutaneously in animals and used to collect bacteria-free wound fluid which accumulates within the cylinders at interface of fibroblastic response. Previous work with guinea pigs (3) indicated that such fluid differed in chemical composition from corresponding blood plasma. It appears likely that time-related changes may occur in content of this fluid during healing. This study was designed to investigate wound fluid throughout 1 to 4 week post-wound period in the dog where larger amounts of fluid are available from single animals.

Materials and methods. Cylinders. All cylinders were made from rectangular pieces of #46 stainless steel surgical foundation wire mesh (60 mm x 30 mm). Each was carefully shaped and secured around 2 lucite rings for support. Male dogs of mongrel, short-haired type weighing about 20 kg were bathed, clipped, and provided with adequate diet. Sterile surgical procedures were employed for implantation and removal of cylinders. Details of surgical method used are reported elsewhere(4). Groups of 16 to 20 cylinders were implanted into subcutaneous tissue along each dog's back. At designated times during healing period (7, 14, 21 or 28 days) cylinders were removed and fluid contents aspirated and pooled. Blood samples were drawn from femoral artery before implantation and before removal of cylinders. In this way changes in wound fluid components could be observed and compared with serum of the wounded animal during all periods. All equipment for collecting fluid was sterilized by autoclaving. Bacteriological checks were made to detect contamination in any samples. The few samples that were bacterially contaminated were discarded. During the first week fibrous tissue encapsulated the cylinders and clear yellow fluid could be aspirated from the interior; about 0.5 ml of fluid/cylinder was present at 5th day after implantation. This increased to a maximum of 2 ml about the 14th day. The volume then decreased as the lining of dense fibro-collagenous connective tissue proliferated inside the cylinder until the 6th week when 1.25 ml of fluid remained. One cylinder which remained in a dog for 8 months still contained nearly 0.5 ml of fluid. Paper strip electrophoretic studies were carried out using Spinco Model R electrophoresis cells by the method of Block, Durrum, and Zweig(5) using .075 M barbital buffer, pH 8.6. Whatman 3 mm paper strips and a current of 5 MA/8 strips were employed. After drying at 110° for 30 minutes, strips were stained with bromphenol blue for protein estimation. For glycoprotein, duplicate strips were stained using periodic acid-Schiff method of Koiw and Gronwall(6) as currently used by Shetlar *et al.*(7). Strips for lipoprotein studies were stained with a saturated solution of Oil Red O in 60% ethanol by the method of Jenck, Durrum, and Jetton(8). A Spinco Analytrol was used for quantitation of dyed strips using #550 interference filter for glycoprotein strips and #500 filter for both protein and lipid strips. Total area under the protein curve was equated to total protein as determined by the biuret method(9), and the glycoprotein curve was equated to total glycoprotein (protein-bound hexose) as estimated by tryptophan method of Shetlar, Foster, and Everett (10). The lipoprotein curve was equated to

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TABLE I. Comparison of Some Constituents of Wound Fluid with Blood Serum during 28-Day Post-Wound Period in the Dog.

Days post-wound	Group	Total protein, g %	Total glycoprotein	Seromucoid	Total lipid	Total phosphatide	Total cholesterol	Free cholesterol
			mg %					
0	Control serum	6.34 ± .12* (18)	121 ± 4 (18)	14.9 ± .3 (3)	607 ± 15 (7)	353 ± 13 (7)	128.3 ± 6.6 (7)	32.2 ± 2.2 (7)
1–14	Serum	6.28 ± .13 (13)	122 ± 5 (13)		615 ± 23 (4)	355 ± 21 (4)	127.0 ± 9.9 (4)	33.1 ± 3.1 (4)
	Fluid	4.02 ± .11† (13)	79 ± 4‡ (13)		327 ± 45 (4)	158 ± 31 (4)	74.8 ± 5.0 (4)	21.4 ± 2.7 (4)
15–28	Serum	6.21 ± .15 (10)	108 ± 6 (10)	14.4 ± .8 (3)	585 ± 29 (4)	337 ± 17 (4)	110.8 ±10.3 (4)	27.5 ± 1.4 (4)
	Fluid	3.28 ± .15† (10)	66 ± 3‡ (10)	12.3 ± .8 (3)	224 ± 25 (4)	94 ± 8 (4)	60.5 ±11.2 (4)	14.5 ± 3.6 (4)

* Mean values ± stand. error; No. of animals studied in parentheses. † Protein significantly decreased between these 2 time periods ($P < .01$). ‡ Glycoprotein significantly decreased between these 2 time periods ($P < .021$).

total lipid content as determined by method of Sperry and Brand(11). Total and free cholesterol were determined using revised method of Sperry and Webb(12), and total lipid phosphorus was done with modification of Youngburg method as described by Hawk, Oser, and Summerson(13).

Results. Wound fluid had significantly lowered amounts of protein, glycoprotein, and lipid when compared to blood serum during the entire period (Table I). In the first 2 weeks after wounding, this fluid contained 64% as much protein, 65% as much glycoprotein, and 53% as much lipid as did serum. Significant decreases were demonstrated between protein and glycoprotein contents of fluid at 1 to 2 weeks and those at 3 to 4 weeks. By 3rd to 4th week post-wound, wound fluid had decreased by 19% in protein, 17% in glycoprotein, and 32% in lipid. Lipid values of fluid when considered relative to protein showed the greatest changes in phosphatide:protein ratio (1:25 at 1-14 days and 1:35 at 15-28 days). However cholesterol:protein ratio (1:54) was relatively unchanged.

All electrophoretically classified groups of proteins found in serum were also present in wound fluid. In some of the electrophoretic fractions, however, quantitative differences were found between fluid and serum. At all times during healing, a greater proportion of albumin was found in wound fluid as compared with serum (Table II). The per cent of alpha-2 and beta-1 globulins in wound fluid was lower than in serum. Total glycoprotein content of fluid showed a smaller decrease during healing than either protein or lipid. Since glycoprotein content did not decrease as much in fluid as did protein, then total glycoprotein to protein ratio increased (Table III). However, the seromucoid fraction of Winzler and Weimer was at the same level (in terms of mg/100 ml of fluid) in both

TABLE II. Electrophoretic Distribution of Proteins in Wound Fluid as Compared to Serum in Dog.

Days post-wound	Group	No. dogs	% albumin	% globulins				
				$Alpha_1$	$Alpha_2$	$Beta_1$	$Beta_2$	Gamma
0	Control serum	18	41.5 ± 1.8*	5.4 ± .3	10.8 ± .6	14.3 ± .7	16.0 ± .9	12.2 ± .9
1–14	Serum	13	41.4 ± 2.2	5.3 ± .3	10.7 ± .7	14.5 ± .7	15.4 ± .7	12.9 ±1.1
	Fluid	13	45.2 ± 1.8	5.8 ± .4	9.8 ± .6	12.7 ± .7	15.8 ± .7	10.8 ±1.0
15–28	Serum	10	43.6 ± 2.6	5.8 ± .1	9.6 ± .4	13.6 ± .7	15.6 ±1.2	12.0 ±1.1
	Fluid	10	48.4 ± 2.3†	6.0 ± .4	8.6 ± .4‡	11.8 ± .7‡	15.2 ±1.2	10.2 ± .7

* Mean ± stand. error. † Significantly higher than in serum at 0-14 days ($P < .02$). ‡ Significantly lower than in serum at 0-14 days ($P < .02$ and $P < .01$).

TABLE III. Electrophoretic Distribution of Glycoproteins in Wound Fluid as Compared to Serum in the Dog.

Days post-wound	Group	No. dogs	Albumin	Bound hexose* associated with Globulins: $Alpha_1$	$Alpha_2$	$Beta_1$	$Beta_2$	Gamma	Total
0	Control serum	18	.46 ±.03†	4.90 ±.34	5.75 ±.25	2.65 ±.14	1.93 ±.09	1.54 ±.11	1.92 ±.06
1–14	Serum	13	.51 ±.04	5.58 ±.36	5.83 ±.29	2.62 ±.14	1.88 ±.17	1.51 ±.12	1.95 ±.09
	Fluid	13	.49 ±.04	5.14 ±.38	6.07 ±.28	3.08 ±.20	1.97 ±.14	1.64 ±.12	1.97 ±.08
15–28	Serum	10	.46 ±.04	4.24 ±.43	5.20 ±.25	2.51 ±.15	1.82 ±.12	1.45 ±.12	1.75 ±.09
	Fluid	10	.59 ±.06	4.92 ±.28	5.88 ±.33	3.47 ±.24‡	2.45 ±.16‡	1.99 ±.16‡	2.03 ±.07

* Expressed as % of protein moiety. † Mean ± stand. error. ‡ Glycoprotein higher in fluid than in serum at this time. ($P < .01$, .01, and .05 respectively.)

fluid and serum (Table I). When corrected for seromucoid content, the wound fluid glycoprotein:protein ratio was similar to that of corresponding serum. Electrophoretic studies also indicated that beta and gamma globulin fractions of wound fluid had comparatively more protein-bound hexose. Table IV shows results from lipid stained strips, which were evaluated in terms of 2 well-defined zones visible called alpha and beta lipoprotein. Wound fluid showed lowered amounts of both fractions compared with the corresponding serum in the 3rd to 4th week post-wound group.

Discussion. Changes in amounts of various components in wound fluid suggest that alterations in permeability for certain molecules in the wounded area have occurred at cell-wall or capillary interfaces. Albumin is significantly elevated in wound fluid, whereas larger protein components, particularly beta and gamma globulins, tend to be lower. The same level of seromucoid in this fluid and serum seems to indicate that this component passes freely from blood stream into the wound fluid in cylinders. Similar findings in comparative studies of lymph and serum of dogs following thermal injury have been reported(14).

TABLE IV. Protein-Bound Lipid Stained with Oil Red O after Paper Electrophoresis.

Days post-wound	Group	No. dogs	Lipid: Expressed as % of total protein in each: Alpha	Beta
0	Control serum	7	6.95 ± .17*	2.29 ± .21
1–14	Serum	4	7.20 ± .37	2.26 ± .39
	Fluid	4	6.99 ± .54	1.25 ± .22
15–28	Serum	4	7.39 ± .34	1.74 ± .18
	Fluid	4	5.66 ± .42†	1.03 ± .16†

* Mean ± stand. error.

† Significantly lower in wound fluid than in serum at this time ($p < .01$ and $p < .04$).

In previous work with guinea pigs(3) a comparison was made of wound fluid with plasma using a moving boundary electrophoresis method. Elevations were found in alpha-2, beta, and gamma globulins of 7th day wound fluid samples. Species differences, or degree of injury to animal, may explain differences noted between the 2 studies.

Elevated glycoprotein values of wound fluid as compared with corresponding serum are of interest. The increase of bound carbohydrate (when expressed as percentage of protein) in the alpha globulin fraction may be explained by comparatively high seromucoid. However, the elevated bound carbohydrate of beta and gamma fractions requires other explanations. One possibility might be that the reparative process utilizes, preferentially, proteins from these fractions with low carbohydrate content leaving those fractions with higher bound carbohydrate. On the other hand, these glycoproteins may be elaborated by cells of the reparative tissue. Additional studies will be necessary to clarify these points.

Summary. Surgical implants of stainless steel mesh cylinders were made into subcutaneous tissue of dog's back. These were removed at intervals of 1 to 4 weeks. Wound fluid was aspirated from inside these cylinders encapsulated with fibrous tissue. When compared with animal's serum, this fluid was lower in protein, glycoprotein, and lipid; and these components decreased with time during post-wound period. As studied by paper elec-

trophoresis, wound fluid had a higher per cent of albumin and lower alpha-2 and beta-1 globulin. An elevation of protein-bound hexose was found in beta and gamma globulin fractions at 3 to 4 weeks. Seromucoid was present at same level in wound fluid and serum. The lipid partition revealed especially low phosphatides but similar cholesterol:protein ratios. It appears that time-related changes occur in large molecular constituents of fluid found in wound area which may be significant in wound healing phenomenon.

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Alterations of Gamma Globulin with Plasma Cell Neoplasm in Mice.* (24938)

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Potter, Fahey and Pilgram described a transmissible plasma cell neoplasm in C3H mice, which was distinguished by production of gamma globulin(1). Nathans, Fahey and Potter measured uptake of *l*-lysine-C^{14} by tumor and demonstrated that gamma globulin formed within tumor was released into general circulation(2). The following investigation was carried out with the intent of further characterizing alterations of plasma protein after partial ablation of tumor and during the course of homologous transplantation of tumor.

Materials and methods. The plasma cell neoplasm, X5563, transfer generation 15, was obtained in its subcutaneous form in 2 living C3H mice.‡ Continuous subcutaneous transmission for 5 generations has been carried out in C3H mice,§ ranging from 6 weeks to 3 months in age at time of transplantation. Subcutaneous transplantation of minced tumor tissue was completed within 1 hour following removal from donor animal. Swiss mice have been used in homologous transplantation experiments. Blood was collected from tail vein in heparinized capillary tubes. Follow-

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§ Obtained from Jackson Memorial Lab., Bar Harbor, Me.

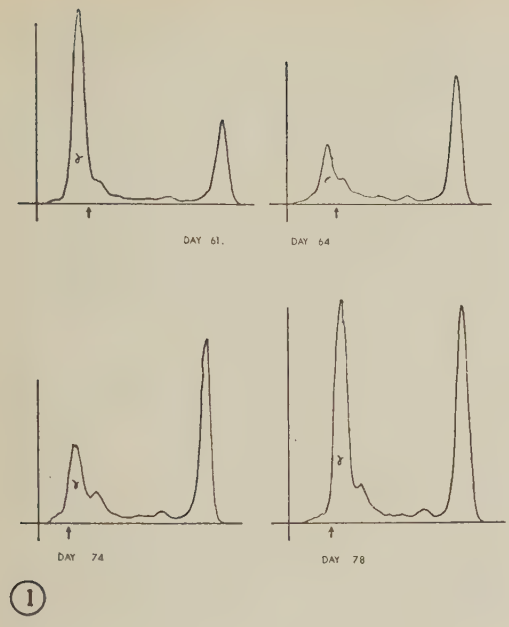

FIG. 1. 1.5 g tumor removed surgically 62 days after transplantation resulted in rapid fall of amount of gamma globulin within 48 hr. Twelve days after removal there was palpable recurrence of tumor and a return of abnormal plasma protein.

FIG. 2. Spontaneous regression of X5563 tumor in mouse given 600 r 2 days before transplantation. On 34th day following transplantation, there was a palpable flank tumor. Characteristic elevation of gamma globulin was present. Twenty-one days later (Day 55) there was a decrease in size of tumor and loss of high gamma globulin peak.

ing centrifugation, the tube was broken and plasma frozen until electrophoresis was carried out. Six lambda (0.006 ml) of plasma was placed on standard Spinco Electrophoretic Apparatus in veronal buffer at pH 8.6. Paper strips were stained with bromphenol blue and graphed on Spinco Analytrol. Total body irradiation was administered from either 250 kv standard therapy unit or Cobalt-60 source of Phoenix Memorial Lab., Univ. of Michigan. Calculated dose was 300 or 600 r.

Results. X5563 is a poorly encapsulated neoplasm composed of relatively undifferentiated cells resembling plasma cells. It metastasizes to bone marrow and spleen. Survival longer than 3 months after subcutaneous transfer is unusual. In all animals in which tumor growth occurred, an abnormal gamma globulin peaking has been demonstrated in plasma by paper electrophoresis. This has been proportional in amount to tumor growth.

Following surgical removal of 0.4 to 1.6 g of subcutaneous tumor in 5 animals, a uniform fall in gamma globulin toward normal levels was noted. A representative experiment is shown in Fig. 1. We have effected no permanent cure by removal of subcutaneous tumor, however, recurrence was delayed in one instance 36 days during which time the electrophoretic pattern was essentially normal.

Ninety-six attempts over 5 generations have been made to transmit X5563 in C3H mice by the subcutaneous route. There were 66 (63%) successful transplantations. All successful transplants were confirmed by development of visible tumor and evidenced characteristic plasma electrophoretic changes. A subcutaneous nodule usually appears within one month of transplantation. The earliest electrophoretic abnormality occurred on 34th day following transplantation. Prior to this time, even in presence of small palpable tumor, no alterations in plasma proteins were seen. In 1 of 6 attempts it was possible to transmit this tumor by subcutaneous transplantation of splenic tissue from an animal sacrificed 74 days following subcutaneous transplant. One tenth ml blood from this and another similar animal injected intraperitoneally in each of 12 mice failed to yield successful transmission.

Homologous transplantation of X5563 tumor into Swiss mice by subcutaneous route

TABLE I. Summary of Attempted Homologous Transplantation of X5563 Tumor.

Group	No. of attempted transmissions	No. of deaths within 30 days	No. of successful transmissions
Non-irrad.	24	5	2
300 r	12	7	1
600 r	21	11	5
Total	57	23	8

has been attempted on 57 occasions. This is summarized in Table I. Total body irradiation was administered 48 hours prior to transplantation. Several animals dying within 30 days manifested hemorrhagic tendencies. Eight of 34 surviving animals developed palpable tumors and electrophoretic plasma changes typical of X5563. From 3 mice in which transplantation was successful, we were able to transmit X5563 to other C3H mice. Four other animals were followed with serial electrophoretic patterns, and in these spontaneous regression of tumor was observed. Fig. 2 illustrates electrophoretic changes occurring in these animals. One animal died of unknown cause.

Discussion. That removal of palpable subcutaneous tumor results in return of electrophoretic pattern to near normal is evidence that source of abnormal globulin is tumor. That this occurs with only partial removal indicates that there is a quantitative relationship between amount of tumor and degree of electrophoretic abnormality.

Observations regarding homologous transplants and tumor regression illustrate genetics of transplantation as outlined by Snell(3). However, successful transplantation and subsequent regression of a tumor have been followed here for the first time without interval sacrifice of host. The number of homologous transplants has been inadequate to determine effect of irradiation, but based on other studies(4,5,6,7), enhanced success in homologous transplantation of X5563 should be expected with prior irradiation of host.

Summary. Experimental mouse plasma cell tumor, X5563, produces plasma gamma globulin alterations. Temporary disappearance of abnormal globulin occurs following partial removal of neoplasm. Homologous transplantation of tumor has been carried out. Because surviving tumor can be readily identified by changes in plasma proteins without animal sacrifice, it provides a new and useful tool for investigation of tumor immunity.

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Decrease of Enzymatic Synthesis of Hexosamine in Epiphyseal Plates of Aminoacetonitrile-Treated Rabbits.* (24939)

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The most evident alterations of experimental lathyrism are in the epiphyseal plates of long bones that appear very widened and lack of cohesion and organization. The changes seem to be due to some defect in ground substance, but there is no agreement about the metabolic block produced by the lathyrogenic agents. An alteration in mucopolysaccharides was suggested some years ago by Ponseti and Shepard(1), and more recently by Pyörälä *et al.*(2). Castellani *et al.*

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(3,4) demonstrated a remarkable decrease of hexosamine, especially galactosamine, in the epiphyseal cartilage hydrolysates of aminoacetonitrile (AAN) treated rabbits. As hexosamine is a major component of mucopolysaccharides, we studied its enzymatic formation in epiphyseal plates of normal and lathyric animals. The synthesis of hexosamine is known to occur in normal growing cartilage of rabbits(5,6).

Methods. Fifteen-day-old rabbits were used. Animals from the same litter, about half were injected subcutaneously with AAN neutralized with Na_2CO_3 daily for 6 days (from 10th through 15th day of life) and killed at least 5 hours after last injection. The others were controls. All animals were killed by decapitation, the legs immediately dissected and put in ice. The epiphyseal cartilage, freed from muscles and periosteum, was excised from the proximal and distal ends of radius, ulna, humerus, tibia and femur and kept in test tube immersed in ice. After weighing, the tissue was homogenized in a Potter-Elvehjem glass homogenizer at 0°C. The homogenizing medium was 10^{-2} M glutamine and 10^{-2} M glucose-6-phosphate Na salt in 0.1 M citrate buffer pH 6.4. The concentration of homogenates was 2.5% since this value was most suitable for enzyme activity(7). Two ml portions of the homogenates were put into 2 Pyrex centrifuge tubes. In one the reaction was immediately blocked by boiling for 10 minutes in water bath and adding HCl to final concentration of 0.3 N. Under these conditions no appreciable hydrolysis of the mucopolysaccharides occurs. After centrifugation at 3000 rpm the supernatant liquid was transferred to a calibrated tube, the precipitate resuspended and washed twice with a little distilled water. The washings were added to the supernatant and the volume made to exactly 3 ml with distilled water. The other portion was incubated for 3 hours at 30°C and thereafter inactivated as described above. The hexosamine was determined on 2 ml portions of these solutions according to Schloss(8). Readings were made at 512 mμ with Beckman DU spectrophotometer taking as blanks the inactivated samples. In some experiments AAN (1 mg/ml) was added to the homogenizing medium of normal cartilage, to test whether or not under these conditions, there was inhibition of enzyme activity. In other experiments adenosintriphosphate (ATP) and uridintriphosphate (UTP) were added to the homogenates of lathyric cartilage to test whether or not the synthesis of hexosamine was enhanced by these compounds. To know if there was any difference in water content between normal and lathyric cartilage, some samples of tissue were dried in oven at 105°C to constant weight.

TABLE I. Hexosamine Synthesized in 3 Hours at 30°C by Epiphyseal Plate Homogenates.

	μg/g of fresh tissue	
	Normal	Lathyric
	1105	159
	1193	282
	1250	239
	1265	346
	1295	361
	1325	346
	1205	316
	1265	376
	1160	241
Avg	1229	296
Decrease		−75.92%

Results. Values obtained for enzymatic synthesis of hexosamine from glutamine and glucose-6-phosphate in normal and lathyric cartilage are shown in Table I. They are expressed as μg of aminosugar synthesized in 3 hours at 30°C per gram of fresh tissue. In 9 experiments here reported, average value for normal cartilage was 1229 μg, and for lathyric cartilage 296 μg/g of fresh tissue, showing average decrease of 75.92%.

In experiments where AAN was added *in vitro* to normal cartilage, we were not able to observe any effect upon synthesis of hexosamine. The synthesis was not enhanced when ATP and UTP were added *in vitro* to lathyric cartilage homogenates.

Dry weights did not show any significant difference between normal and lathyric cartilage.

Discussion. Our results and those of Castellani *et al.*(3,4) suggest that alterations in ground substance of the epiphyseal plate in AAN induced lathyrism are due to a defect

in metabolism of mucopolysaccharides.

The enzymatic reaction we studied (glutamine + glucose-6-phosphate → glutamate + hexosamine-6-phosphate) was first demonstrated by Leloir and Cardini in *Neurospora crassa*(9). The involved enzyme, called hexosamine synthetase, occurred in the epiphyseal plates of rabbits(5,6), in bone callus of experimental healing fractures(10), and to a minor extent in other tissues(6,11). Pogell recently confirmed its occurrence in rat liver (12,13). The aminosugar formed was incorporated in a complex, probably chondroitinsulphate(7). Little is known about this enzyme. Several compounds were tested which showed no coenzyme activity(9,12). In some of our experiments, when ATP and UTP were added *in vitro* to the lathyric cartilage homogenates, we could not obtain evidence that these compounds act as limiting factors for the synthesis. The importance of hexosamine synthetase in relation to metabolism of mucopolysaccharides and mechanism of bone formation was emphasized by Zambotti(14).

The nature of the action of AAN upon synthesis of hexosamine in the experimental animals remains to be elucidated. As we obtained no evidence of any effect when AAN was added *in vitro* to normal cartilage, we cannot suppose a direct inhibition of the enzyme. We do not know if the AAN acts upon the existing enzyme by some product of its metabolism, by inhibition of enzyme formation, or by destruction of the same. Castellani and Castellani-Bisi(3) reported that the amount of total collagen, as determined by hydroxyproline content, is the same in normal and lathyric epiphyseal plates. This finding was confirmed by Follis and Tousimis(15) who also, in a study with the electron microscope, were able to demonstrate that the collagen extracted from epiphyseal plates of AAN treated animals is not organized in fibrils, as it normally is. We do not know how the collagen organizes itself into fibrils *in vivo*, but there is evidence(16,17) of a linkage between collagen and mucopolysaccharides. An alteration in quantity or quality of the latter could perhaps explain lack of formation of collagen fibrils.

Summary. Enzymatic synthesis of hexosamine from glutamine and glucose-6-phosphate in epiphyseal plates of normal and lathyric rabbits has been studied. In the cartilage from experimental animals a decrease of 75.92% of this synthesis was found. AAN added *in vitro* did not have any inhibitory effect upon formation of hexosamine.

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Hemolysin Formation in Mice Following Partial, Total Splenectomy or Spleen Transplantation. (24940)

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Importance of the spleen in fixation of antigens(1) and in regulation of antibody formation(2,3) has long been recognized. Current interest in immunologic problems encountered in foreign tissue transplantation experiments, especially in radiation protection investigations(4,5), stimulates the search for methods of reducing unfavorable host reactions. The present study was undertaken to determine the contribution of spleen to primary hemolysin response of mice.

Methods. Surgical removal of part or all of the spleen was done within 24 hours of birth in mice of both sexes of inbred NIH strain. Other groups of male mice of same strain were similarly partially splenectomized at 11 weeks of age. Sham surgical operations were performed on littermate animals of both ages for control purposes. Veterinary nembutal (0.3 mg/g body weight) was used as anaesthetic for older animals and ether for infant mice. In transplantation studies, described in detail later, splenectomized mice of 2 unrelated strains, NIH and Balb/c, served both as donors and recipients of whole spleens, each cut into 4 pieces. Splenectomy was immediately followed by transplantation of tissue into peritoneal cavity of recipients and immunization was done 21 days later. Mice were inoculated intravenously or intraperitoneally at 12 to 15 weeks of age with 2.5% suspension of sheep erythrocytes given as 0.01 ml/g of body weight. Serum was collected at sacrifice 5 days after immunization and the serum hemolysin content was estimated colorimetrically as described by Taliaferro(6). Spleens from control mice, spleen tissue fragments and remnants of spleens of partially splenectomized mice were weighed after blotting at time of bleeding.

Results. From the trend indicated by distribution of individual serum hemolysin titers shown in Fig. 1 it is concluded that the capacity of mice to form antibody was interfered with when the amount of remnant spleen was reduced below .2 to .3% of body weight. Average spleen weight for 11 mice (avg. wt. = 28.9 g) given sham splenectomy as adults was 154 ± 13 mg (S.E.) and for 25 mice (avg. wt. = 25.8 g) similarly treated within 24 hours of birth was 122 ± 3 mg. Corresponding average hemolysin titers were −2.6 ± .03 and −2.7 ± .1 respectively. Removal on the average of more than 45 to 55% of spleen tissue in mice was associated with reduced capacity for hemolysin formation.

Total splenectomy in NIH mice completely suppressed hemolysin formation when the antigen was given intravenously 21 days after surgery. Results of independent experiments demonstrated that average hemolysin titers of groups of mice of 5-10 animals/group, bled 6 days after immunization on day of splenectomy or 7 or 14 days thereafter, did not differ significantly from average titers observed in mice immunized at 21 days nor was there any detectable change in time required for development of peak titer. A reduction both in rate and amount of antibody formed was observed following intraperitoneal immunization of splenectomized mice because the average hemolysin titer in 9 intact mice on 5th day after antigen inoculation was −2.68 ± .1 (S.E.) compared to only −1.97 ± .1 for 10 splenectomized mice 6 days after immunization. It is of interest to note that in other experiments increases to 30% over average control values in the amount of spleen tissue by transplantation of isologous spleen to groups of otherwise intact Balb/c mice were ineffective in increasing their average hemolysin titers.

Since transplanted pieces of spleen did not increase hemolysin titers of mice, observations were made on the effect of transplanted spleens of autologous, isologous or homologous origin on hemolysin formation of splenectomized recipients. Table I gives average se-

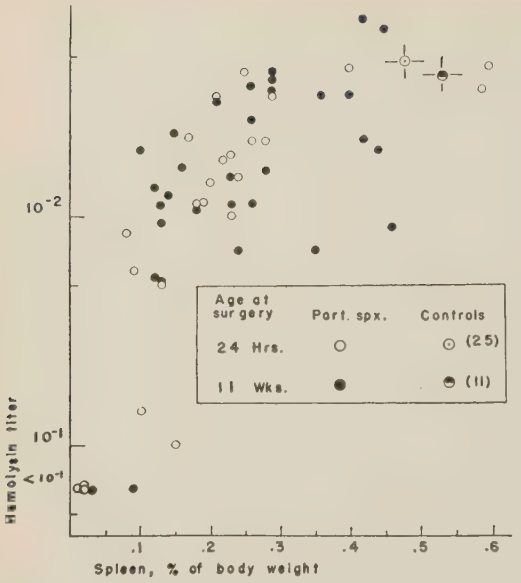

FIG. 1. Individual serum hemolysin titers of mice with the indicated amounts of spleen tissue compared to the averages observed in corresponding sham-operated controls. Numbers in parentheses indicate numbers of control mice/group and bars represent 95% confidence limits of the means.

rum hemolysin titer 5 days after a single intravenous immunizing injection made 21 days after splenectomy and spleen transplant in 2 strains of mice. When the mouse spleen was removed, cut into 4 pieces and returned to peritoneal cavity of same animal, the 5th day titers averaged –1.24 and –1.87 for 25 NIH and 13 Balb/c mice respectively. Average titers were significantly lower when isologous transplants were made in both strains as shown in Table I and homologous transplants were associated with a complete suppression of the hemolysin response. A few NIH mice which received their own spleens as suspensions of cells were unable to form hemolysin after intravenous immunization. Gross observation of the condition of transplanted pieces of spleen supplied evidence that in general good "takes" with apparently rich vascularization were associated with antibody formation. However, isologous transplants in 7 of the 18 NIH mice were judged as good "takes" at autopsy, but only 2 of these mice formed detectable amounts of hemolytic antibody. All but one of the 13 Balb/c mice which received isologous spleens had healthy appearing transplants and this animal failed to produce hemolysin on challenge. This discrepancy between the 2 strains may be attributed to genetic differences arising from breeding procedures.

TABLE I. Hemolysin Formation in 2 Strains of Splenectomized Mice Receiving Whole Spleen Transplants of Autologous (A),* Isologous (I)* or Homologous Origin.

Strain		No.	Avg hemolysin† titer
Donor	Host	mice	± S.E.
NIH controls		15	$-2.77 \pm .04$
Balb/c controls		10	$-2.91 \pm .05$
NIH	NIH(A)	25	$-1.24 \pm .19$
Balb/c	Balb/c(A)	13	$-1.87 \pm .09$
NIH	NIH(I)	18	$16 = <-1.0; -1.25, -2.45$
Balb/c	Balb/c(I)	13	$-1.49 \pm .14$
NIH	Balb/c	10	$< -1.$
Balb/c	NIH	10	"
NIH	NIH(A)‡	5	"

* Spleen was surgically removed, cut into 4 pieces and returned to the peritoneal cavity of same animal (A) or another animal (I).

† Serum hemolysin was estimated at 5 days after intrav. immunization 21 days after splenectomy and transplant.

‡ Each spleen was removed, macerated in Potter type homogenizer in Tyrode's solution and resulting cell suspension intrav. injected.

From the observation that antibody titers averaged only about 27% less and rate of production was somewhat reduced in splenectomized mice given antigen by intraperitoneal route, it is suggested that spleens of intact mice served chiefly as filter organs for the particulate antigen. This possibility was substantiated by the observation that transplanted autologous spleen cell suspensions did not improve hemolysin titers of splenectomized NIH mice while at least some antibody was formed in mice with transplanted splenic tissue characterized by good "takes."

Summary. Serum hemolysin titers were correlated with weight of remnant spleen present in groups of partially splenectomized NIH mice sacrificed 5 days after intravenous immunization with sheep erythrocytes. Total splenectomy of mice completely suppressed hemolysin formation after intravenous immunization and was associated with a 27% decrease in amount of antibody formed, and a 24 hour delay required for development of peak serum titer. Complete suppression of antibody formation was partially reversed by

autologous spleen transplants in splenectomized mice of 2 strains; by isologous transplants in Balb/c but not in NIH mice; and unaffected by homologous transplants in either of the 2 strains.

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Neonatal Bilirubin Levels Following Administration of l-Triiodothyronine.* (24941)

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Association of prolonged jaundice with cretinism was noted by Christensen(1). He suggests that lack of thyroid hormone may delay hepatic enzyme maturation just as it delays maturation of ossification centers. When Christensen gave thyroid hormone to cretinous infants, there was a prompt fall in their serum bilirubin. His observations prompted us to undertake a study of the effect of thyroid substance on physiologic hyperbilirubinemia of the newborn. l-Triiodothyronine† was selected for administration to infants because of rapidity of its action.

Methods. Using the system of alternates, 100 test and 100 control infants were selected upon admission to nursery. All infants were normal, full term and were admitted to nursery from delivery. Each test infant was given oral dose of 25 μg of l-triiodothyronine daily for 4 days. Control infants were given no medication. Bilirubin determinations were done on each infant at 24-48 hours and 72-96 hours of age. Blood samples were drawn during morning hours by heel puncture technic. Bilirubin was measured by method of Hsia (2) as modified by C. C. Roby (personal communication). Levels of direct bilirubin were low, hence only total bilirubin was considered in calculations. The effect of l-triiodothyronine on bilirubin disposition was evaluated by comparing mean difference between first and second bilirubin levels in test and control groups, by comparing means of 24-48 and 72-96 hour specimens in test group with corresponding means in control group, and by comparing number of infants in each group who showed a rise in serum bilirubin during interval between the 2 determinations. Red cells of all infants were tested by direct Coombs technic for presence of blocking antibodies. ABO and Rh typings were done on all infants and their mothers.

Results. The mean difference between first and second bilirubin determinations in babies to whom l-triiodothyronine was given, was $+ 0.53 \pm 0.28$ mg/100 ml. The mean difference between the 2 specimens in control group was $+ 1.6 \pm 0.31$ mg/100 ml ($t = 7.0$; $p<0.01$). There was no evidence that the 2 groups differed in variability. Mean bilirubin level in test infants at 24-48 hours was 5.0 ± 0.25 mg/100 ml, range 0.8-12.2 mg/100 ml and at 72-96 hours was 5.5 ± 0.41 mg/100 ml, range 0.0-16.5 mg/100 ml. In control infants, the mean for first specimen was 5.8 ± 0.26 mg/100 ml (range, 0.8-13.4 mg/100 ml) and for second specimen, 7.3 ± 0.45 mg/100 ml (range, 0.6-19.3 mg/100 ml)

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† 1-triiodothyronine (Cytomel®) was generously supplied by Smith, Kline & French Lab.

TABLE I. Serum Bilirubin Levels in mg/100 ml at 24-48 Hr and 72-96 Hr of Age in Infants Given 25 μg of l-Triiodothyronine and in Controls.

	l-Triiodothyronine	Control*	p
Avg bilirubin, 24-48 hr	5.0 ±.25	5.8 ±.26	<.01
Idem 72-96 "	5.5 ±.41	7.3 ±.45	"
Avg diff. in bilirubin level between 1st and 2nd specimens	.53±.28	1.6 ±.31	"

* 100 infants.

(Table I). Forty-three of 100 test infants and 64 of 100 control infants demonstrated a rise in bilirubin during period between first and second determinations. $Chi^2 = 8.86$; p 0.005 (Table II). There was no evidence of bilirubin encephalopathy in any infant in the study.

The direct Coombs test was negative on all babies (Table III). There was a potential ABO incompatibility in 25 control and 22 test infants. This difference is not significant ($chi^2 = 0.25$; $0.5<p<0.7$).

Discussion. These results indicate that l-triiodothyronine exerts a depressant effect on serum bilirubin level of the newborn. This finding is consistent with Christensen's observation of the effect of thyroid hormone on prolonged neonatal jaundice in athyrotic infants. It is somewhat surprising in light of elevated levels of serum butanol extractable iodine found in newborns by Man(3) and Pickering(4). These investigators found neonatal BEI markedly elevated, reaching a peak at about 4 days of age. Van Middlesworth(5) observed an elevated I^{131} uptake during newborn period and suggested that newborn infants may undergo a period of physiologic hyperthyroidism.

The avidity of neonatal thyroid gland for iodine might also be increased by lack of thyroid substance in a form available for utilization. The elevated serum BEI might then be a reflection of the presence of thyroxine-like substances which are tightly bound by protein or otherwise inactivated and not available to the tissues.

TABLE II. Direction of Change of Bilirubin Level between 24-48 Hr and 72-96 Hr of Age in Control Infants and in Infants Given 25 μg of l-Triiodothyronine per Day.

	Infants with rising bilirubin	Infants with unchanged or falling bilirubin	Total
l-Triiodothyronine	43	57	100
Controls	64	36	100

$Chi^2 = 8.86$; $p < .005$.

Kurland(6) has suggested that tissues are more permeable to l-triiodothyronine than to thyroxine. It may be that in the newborn l-triiodothyronine can relieve a cellular deficiency of thyroid hormone and speed maturation of glucuronic acid conjugating system. Peterson(7) has shown an accelerated reduction and conjugation of compound F in thyrotoxic adults. Similarly in infants, excessive thyroid substance may speed the disposition of bilirubin.

Another possible explanation of our results is suggested by the effect of sulfisoxazole on serum bilirubin. Harris(8) found lowered bilirubin levels and marked increase in incidence of kernicterus in a group of premature infants who were given sulfisoxazole. Johnson (9) administered sulfisoxazole to rats congenitally deficient in glucuronyl transferase. The drug lowered the rats' bilirubin, increased the incidence of bilirubin encephalopathy and caused a marked intensification of bilirubin staining in fat and nervous tissue. Thus, it may be that l-triiodothyronine accomplishes a redistribution rather than an increased excretion of bilirubin.

TABLE III. Incidence of Possible Major Blood Group Incompatibility in Test and Control Infants.

	ABO incompatible	ABO compatible	Total
l-Triiodothyronine	22	78	100
Control	25	75	100

$Chi^2 = .25$; $.5 < p < .7$.

The decrease in serum bilirubin response to the dose of l-triiodothyronine used, was not large enough to be of clinical value. Furthermore, we have not eliminated the possibility that administration of the drug may increase rather than decrease incidence of bilirubin encephalopathy. Therefore, we do not recommend clinical use of l-triiodothyronine to control icterus neonatorum.

Summary. The effect of l-triiodothyronine on serum bilirubin in the newborn was

studied. The mean difference between bilirubin levels at 2 and 4 days of age was determined in 100 infants given 25 μg of l-triiodothyronine daily. This mean was compared with a similar mean in 100 controls. l-triiodothyronine significantly depressed the neonatal bilirubin level. The drug may exert its effect by accelerating maturation of glucuronic acid conjugating system.

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Diabetogenic Action of Analogues of 8-Hydroxyquinoline. (24942)

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As previously reported(1,2,3), some chelating agents for heavy metals have been shown to be diabetogenic in experimental animals. This effect may be attributed to their destructive action on the pancreatic islet cells by chelating with biologically important metallic ions. Further studies have been continued to detect other diabetogenic chelating chemical substances with structural similarity to 8-hydroxyquinoline. The present report deals with studies on the diabetogenic action of analogues of 8-hydroxyquinoline and derivatives of pteridine and similar structures with metal-binding properties.

Materials and methods. Adult rabbits were used. Blood sugar was determined by Hagedorn-Jensen's method(4) at various intervals after intravenous administration of the compounds. Histologic examination of pancreatic islet cells was performed with Gomori's chromium-hematoxylin phloxine method(5). Analogues of 8-hydroxyquinoline were used as solutions of sodium salts. Derivatives of pteridine were administered as aqueous solutions. Tested compounds and intravenously injected doses were as follows: 1-Hydroxyphenazine 30-60 mg/kg, 6-Hydroxy-m-phenanthroline (5:6-Pyridooxine) 30-70 mg/kg, l-Hydroxyacridine 20 mg/kg, 5-Hydroxybenzo-f-quinoline (5:6-Benzooxine) 30-50 mg/kg, 5, 8-Dihydroxyquinoxaline 50 mg/kg, 4-Hydroxypteridine 20 mg/kg, Isoxanthopterine 20 mg/kg, 2-Amino-4-hydroxypteridine 15-30 mg/kg, Picolinic acid 40-100 mg/kg, Dipicolinic acid 20-40 mg/kg.

Results. 1-Hydroxyphenazine caused a transitory hyperglycemia. However, doses over 60 mg/kg were intolerable, as they showed considerable toxicity. 1-2 hours after injection with doses 50-70 mg/kg of 6-Hydroxy-m-phenanthroline there ensued a remarkable hyperglycemia which returned to normal after 3-4 days (Fig. 1). Histologic examination of the pancreas of one rabbit which died 24 hours after injection revealed necrotic and degenerative changes of beta cells of the islets.

1-Hydroxyacridine also could produce a hyperglycemia as shown in Fig. 1. One rabbit died in convulsion 24 hours after administration of 1-hydroxyacridine (blood sugar 26 mg/100 ml). Histologic appearance of the islet of this animal showed degenerative changes of beta cells, while alpha cells remained almost normal. 5-Hydroxybenzo-f-quinoline caused similar hyperglycemia. However, degree of hyperglycemia was somewhat slighter than those produced with above 2

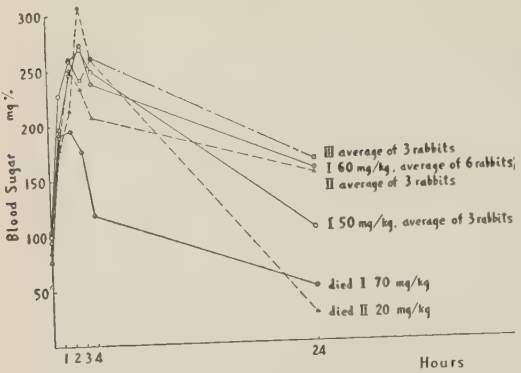

FIG. 1. Changes in blood sugar level in rabbits after intrav. administration of analogues of 8-hydroxyquinoline. I. 6-Hydroxy-m-phenanthroline, II. 1-Hydroxyacridine, III. 5-Hydroxybenzo-f-quinoline.

compounds. After administration of 5,8-dihydroxy-quinoxaline no fluctuation of blood sugar was observed. Three derivatives of pteridine and dipicolinic acid caused no noticeable change in blood sugar with given doses. Picolinic acid showed only a slight hyperglycemic tendency.

Discussion. For elucidating the acting mechanism of diabetogenic substances, it may be important to study the correlation between chemical structures and biological action. Selective affinity of chelating agents for zinc in the islet cells seems to be a clue for the problem. The data presented above show clearly the diabetogenic action of 1-hydroxyacridine, 6-hydroxy-m-phenanthroline and 5-hydroxybenzo-f-quinoline. These compounds have sensitive chelating activity for trace metals, as shown by Albert(6). Therefore, the action of these compounds is presumed to be a result of chelation with zinc ions in the islet cells. Despite its sensitive chelating avidity(7), 5,8-dihydroxy-quinoxaline had no effect on blood sugar. It may be considered, as in quinoline derivatives(3), that addition of hydroxy groups in the compound deprives it of the biological action.

Considering analogous structures to 8-hydroxyquinoline and chelating activity of pteridine derivatives(8), diabetogenic action of some derivatives of pteridine was expected. However, these compounds did not affect blood sugar. Hörlein(9) reported that intravenous administration of xanthopterine into rabbits in doses 20-50 mg/kg caused destruction of kidney and hyperglycemia during a few days after injection. Albert(8) showed that 4-hydroxypteridine, in spite of its structural similarity to 8-hydroxyquinoline, had a far lower affinity for metallic ions. The lack of diabetogenic effect of pteridine derivatives might have been due to poor solubility and lower chelating affinity for metals.

Despite their chelating affinity(10,11), picolinic acid and dipicolinic acid had no diabetogenic action, probably due to the presence of the carboxyl group, as in the case of quinaldinic acid(1). Therefore, available evidence indicates the existence of the essential chemical structures and properties required for diabetogenic action. Besides chelating affinity for trace metals, suitable solubility and property seem to be essential. Much more evidence may be necessary to disclose the possible mechanism of diabetogenic substances.

Summary. 6-Hydroxy - m - phenanthroline and 1-hydroxyacridine caused hyperglycemia and destructive changes of the pancreatic islet in rabbits. 5-Hydroxybenzo-f-quinoline also produced a considerable hyperglycemia. However, 3 derivatives of pteridine, picolinic acid and dipicolinic acid failed to produce significant changes in blood sugar.

We are greatly indebted to Dr. F. Hirata, Ono Pharmaceutical Co., Ltd. for synthesis of compounds used.

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Chemotherapy of Ehrlich's Ascites Tumor with Cyanide and Ether Combinations.* (24943)

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The early research of Warburg indicates that a general biochemical characteristic of tumors as opposed to normal tissues is their high rate of anaerobic fermentation accompanied by low efficiency of oxidative phosphorylation(1). Warburg contended that this is the result of irreversible impairment to oxidative phosphorylative capacity of the original malignant cell during carcinogenesis, and that oxidative metabolism in malignant cells may approach an irreducible minimum. Weinhouse showed however, that damage to oxidative capacity of the tumor cell is not as clear cut as seems from Warburg's data(2). He demonstrated that CO_2 production from glucose or fatty acids is similar in malignant cells and normal tissues. In addition it has been shown that effects of inhibitors such as fluoride or dinitrophenol are similar in normal tissues and tumors(3). Quastel showed that respiration and phosphorylation in tumor cells are tightly coupled(4). If Warburg's theory is accepted as a working hypothesis the large quantitative differences in oxidative capacity between normal and tumor cells could form an excellent basis for chemotherapy. Use of the cyanide ion in cancer chemotherapy is not new. Older research demonstrated that this ion seemed to have a differential inhibitory effect upon malignant tissues(5,6,7), but the margin of safety appeared so low that interest was lost. Our data represent the effects of sublethal doses of cyanide given to tumor bearing animals under the influence of ether anesthesia.

Methods. Our animals were male Swiss mice obtained from Taconic Farms. Weight averaged 20 g $\pm$ 10%. The animals were injected intraperitoneally with Ehrlich's ascites tumor cells obtained from stock inoculated 8 days previously. The cells were withdrawn under light ether anesthesia using No. 18 needle and 2 ml syringe. Tumor cells were centrifuged 2 minutes in a centrifuge, peritoneal fluid was decanted, and the cells resuspended in 10 $\times$ dilution of normal saline, and immediately injected. Therapy was reserved in Group 1 until ascites became grossly evident at fifth day post inoculation. Control and experimental animals were selected from inoculated mice at random. Treatment was as follows: Experimental mice were subjected to an anesthetic concentration of ether in a bell jar, and when no longer responsive to painful stimuli were injected intraperitoneally with 0.75 or 1.5 mg/kg of sodium cyanide in normal saline. The volume injected was 0.01 ml/10 g of mouse. Animals were allowed to recover from ether anesthesia. Control animals were not subjected to sham procedure with ether and normal saline because this regimen slightly decreased survival time of animals. Time intervals from inoculation to institution of treatment and doses were varied from group to group.

Results. In Group 1 an extremely large inoculum was utilized, (8×10^7 cells), and treatment was established, as schematically represented in Fig. 1 and Table I. In addition the control group consisted of 40 animals as opposed to 10 experimental mice.

Group 2 is represented in Fig. 2 and in Table I. Treatments were instituted at times noted on Fig. 2 and in the Table. There were 10 mice/experimental procedure.

Group 3 is presented in Table I and Fig. 3. Dosage was increased to 1.5 mg/kg NaCN. Treatment was instituted at 24, 48, and 120 hours post inoculation. A further group also received multiple treatment on first, third, and fifth day after implantation of tumor. Ten mice were used in each condition.

Discussion. These data demonstrate that significant increases in survival time of mice

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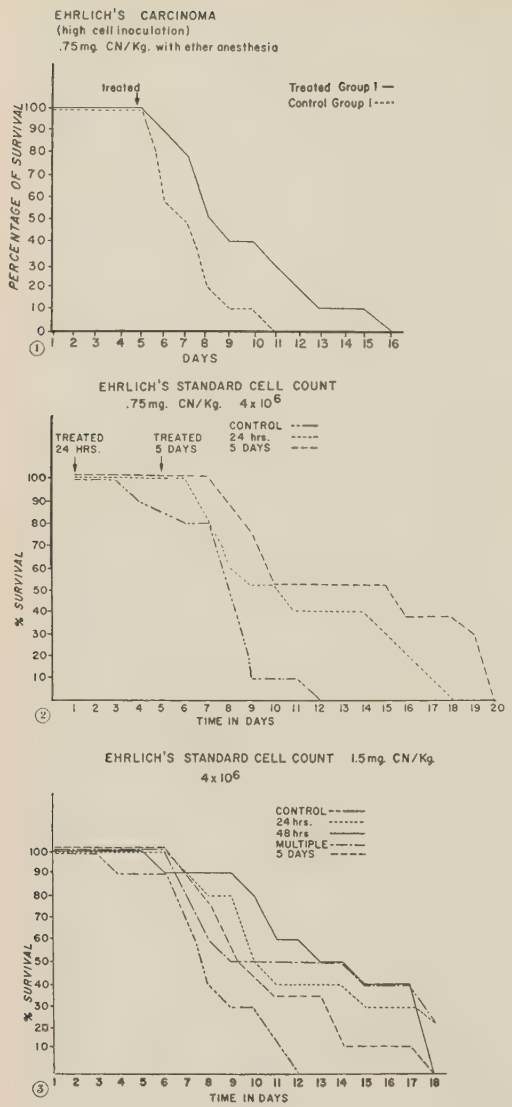

FIG. 1. Survival curves for Group I showing comparison between control Ehrlich's ascites mice and animals treated with 0.75 mg/kg cyanide and ether anesthesia (8×10^7 cell inoculation).

FIG. 2. Survival curves for Group II (4×10^6 cell inoculation) showing comparison between control animal survival and that of animals treated at 24 hr, and others treated at 5 days post inoculation with 0.75 mg/kg sodium cyanide and ether anesthesia.

FIG. 3. Group III (4×10^6 cell inoculation) survival curves comparing survival of control animals with that of animals treated at 24 hr, 48 hr, 5 days, and a series treated at 1, 3, and 5 days post inoculation with 1.5 mg/kg cyanide and ether anesthesia.

with Ehrlich's ascites carcinoma may be obtained with sodium cyanide and ether as chemotherapeutic agents. We consider cyanide-ether combinations superior to cyanide alone because our pilot studies demonstrated 57% average increase in survival time over animals given cyanide alone. In addition, we demonstrated a decrease in acute toxicity of cyanide under anesthesia which will be reported elsewhere.

As to the mechanism of action of these agents, these data do not give definite answers. Several possibilities may be pointed out. The action of cyanide may be a corollary of Warburg's hypothesis. However, it should be pointed out that cyanide blocks many enzyme systems, and although inhibition of cytochrome oxidase is the most evident of these actions, inhibition of another enzyme system may be responsible for increase in survival time. Chance has shown that addition of glucose to Ehrlich's ascites causes rapid acceleration of respiration, and that reduced pyridine nucleotide, flavoprotein, cytochromes *b* and *c* and in some cases cytochrome *a* are more oxidized during glucose activated phase of respiration(8). It is suggested that the well known hyperglycemic action of ether may be related to synergism between cyanide and ether in treatment of this tumor. The respiratory enzymes thus activated by release of endogenous glucose may then be more vulnerable to the action of the cyanide ion. In addition the use of ether may extend the margin of safety in use of cyanide. The possibility that malignant cells killed by cyanide may serve as antigenic material also exists. Finally, it should be noted that Ehrlich's ascites tumor can be inhibited by a variety of compounds seemingly unrelated in mechanism of action or chemistry(9).

Experiments involving the combination of cyanide, ether, and inhibitors of DNA synthesis, and also the use of cyanide and ether in treatment of spontaneous tumors of dogs are in progress.

Summary. It has been demonstrated that sodium cyanide given intraperitoneally at several sublethal dosage levels during ether anesthesia is capable of significantly prolonging survival time of mice inoculated with Ehrlich's ascites carcinoma.

TABLE I. Alteration of Survival Time in Ehrlich's Ascites Mice Produced by Cyanide-Anesthesia Treatment.

Group No.	Dosage (mg/kg)	Cells/mouse	Avg survival (days)	Increase in survival (days)	% increase
I Control	.75	8×10^7	6.2	0	0
5 days	"	"	8.8	2.6	42.0
II Control	.75	4×10^6	8.1	0	0
24 hr	"	"	9.8	1.7	21.0
120 "	"	"	10.4	2.8	35.0
III Control	1.5	"	8.1	0	0
24 hr	"	"	12.7	4.6	56.8
48 "	"	"	13.8	5.7	73.7
120 "	"	"	11.1	3.0	37.0
27, 72, 120 hr	"	"	12.8	4.7	64.1

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Adrenal Cortical and Body Temperature Responses to Repeated Endotoxin Administration. (24944)

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It is difficult to speculate logically on the mechanism of adrenal cortical stimulation following endotoxin, inasmuch as fever and hypotension may be responsible. Previous studies indicate that the adrenal cortical stimulating effect occurs before fever(1), but most animals that reveal pituitary-adrenal activation following endotoxin also develop fevers. The present studies were set up to produce tolerance to fever-promoting effect of endotoxin. The ease of producing tolerance to various responses following endotoxin varies considerably, and it was possible that fever-tolerance might occur before loss of pituitary-adrenal stimulation.

Methods. Adult mongrel dogs ranging 8-25 kg were used. Cannulation of right lumbar vein(2) was performed according to the technic of Hume and Nelson, and intermittent samples of adrenal venous blood were obtained. Cortisol concentrations in the adrenal venous blood were determined by modification of method of Silber and Porter(3) as described by Peterson, Karrer and Guerra(4). Purified endotoxin of Boivin type derived from *E. coli* was prepared by method of Spink and Anderson(5). Endotoxin was prepared in 2 concentrations in normal saline, one of 1 mg/cc, the other 0.1 mg/cc. All endotoxin injections were by intravenous route and given in approximately 30 seconds time. Twenty-five units of ACTH were given intravenously at conclusion of some experiments. Rectal temperatures were taken, and in some experiments arterial blood pressures were obtained by cannulating femoral artery with polyethylene catheter connected to mercury manometer.

Results. The adrenal cortical and fever responses to large doses of endotoxin are given in Table I. Significant adrenal cortical responses were observed in all animals except

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TABLE I. Effect of Repetitively Administering Large Doses of Bacterial Endotoxin on Body Temperature and Adrenal Cortical Function.

Exp. No.	Days of previous endotoxin exposure	Dosage of endotoxin (mg)	Adrenal venous blood 17-hydroxycorticosteroids (μg/min.) Time after endotoxin (min.) Control	5	60	120	180	Rectal temp. elevation (°C)
R-1	3	4.0	1.8	10.0	16.3	15.8	1.1	1.9
R-2	5	4.0	1.6	11.4	10.2	5.3	11.6	2.5
R-3	3	.5	.7	2.7	6.9	1.9	1.6	2.2
R-4	3	.1	.7	2.2	1.0	.9	1.5	.3
R-5	3	.1	.3		6.0	5.7		N.D.*
R-6	3	.1	3.2	3.6	5.4	7.6	9.9	2.2
R-7	3	.1	.5	.8	5.6	5.2		N.D.
R-8	3	.1	.7	15.9	13.1	15.1	1.7	2.0

* N.D.—not determined.

R-4, with dosages ranging 0.1 to 4 mg of endotoxin/day, and for periods to 5 days. Except for R-4, no instance of tolerance to fever-stimulating effects of endotoxin were observed.

In Table II are given results following administration of 0.01 mg of endotoxin for 1 or more days prior to collecting adrenal venous blood samples. In none of the 8 experiments was an elevation in rectal temperature or hypotension observed, although all animals had rectal temperature elevations of at least 1.5° and significant adrenal cortical responses on first day of endotoxin administration. Although all animals manifested tolerance to fever-stimulating effect of endotoxin, only one animal, ER-15, was completely tolerant to adrenal cortical-stimulating effect. All other animals demonstrated significant adrenal cortical responses following administration of endotoxin, although this response was not maximal when compared to that following intravenous ACTH. In some experiments the differential between maximal levels of cortisol output following endotoxin and that following ACTH was less than in others.

Experiments were then performed in which endotoxin was administered on consecutive days, with time intervening before final injection, Table III. In ER-1, a maximal adrenal cortical response and significant fever was observed following administration of endotoxin. On second day of endotoxin administration a significant adrenal cortical effect was observed, but no fever. At 60 days fever tolerance had been lost. Similar findings were observed in Exp. ER-4 and ER-5, in which fever responses returned at 43 and 36 days respectively. In Exp. ER-12 fever tolerance existed on second and third day following administration of 0.01 mg of endotoxin, but 3 days later normal fever response had been restored, Fig. 1. Adrenal cortical response was significant on days 2 and 3, in absence of fever. The response was not maximal as shown by increase in corticoid output follow-

TABLE II. Effect of Repetitively Administering 0.01 mg of Bacterial Endotoxin on Body Temperature and Adrenal Cortical Function. All animals had rectal temperature elevations of at least 1.5°C and significant adrenal cortical responses on 1st day of endotoxin administration.

Exp. No.	Days of previous endotoxin exposure	Adrenal venous blood 17-hydroxycorticosteroids (μg/min.) Time after endotoxin (min.) Control	60	90	120	5 min. after I.V. ACTH	Rectal temp. elevation (°C)
ER- 2	1	1.3	11.9		9.5	14.4	+.6
ER- 3	1	.2	4.6	6.1	7.1	14.0	−.1
ER- 6	1	3.7	9.5	12.0			No change
ER- 7	1	1.8	6.7	6.1	6.0	14.1	+.2
ER-14	5	.2	6.3	8.9		18.3	+.4
ER-15	5	.5	.2	1.8	.5	9.1	No change
ER-16	6	.4	3.8	4.8	6.8	13.9	"
ER-17	6	.4	3.6	8.9	9.2	25.8	"

TABLE III. Adrenal Cortical and Body Temperature Responses of Dogs Given 0.01 mg of Endotoxin on Consecutive Days, Followed by Later Endotoxin Administration.

		Adrenal venous blood 17-hydroxycorticosteroids (μg/min.)					
			Time after endotoxin (min.)				
Exp. No.	Day of endotoxin admin.	Control	60	90	120	5 min. after I.V. ACTH	Rectal temp. elevation (°C)
ER- 1	1	1.9	10.5	10.4	11.6	8.1	+1.6
	2	.4	8.1			7.1	− .2
	60	.7	9.6	11.1			+1.8
ER- 4	1	.3	7.5	7.7	13.7	12.5	+1.6
	2	1.2	4.5	2.4	10.0	8.5	+ .2
	43	.4	5.2	8.1	9.8	8.5	+4.0
ER- 5	1	2.7	3.9	6.1	5.6	8.1	+ .9
	2	1.4	3.7	6.7	6.0	12.6	− .3
	36	.6		4.5	6.1		+2.9
ER-12	1	.8	9.1	8.0	16.8	14.4	+2.8
	2	.4	4.5		8.8	19.3	+ .1
	3	.3	9.7	7.9	9.0	13.6	+ .4
	6	.2	6.6	11.5	13.8	16.7	+3.1

ing administration of intravenous ACTH. Normal fever and adrenal corticoid responses had returned by 6th day.

Discussion. Our results indicate that tolerance to fever-promoting effects of endotoxin may be induced more readily than to adrenal cortical stimulating properties. In many experiments partial tolerance to adrenal cortical stimulating properties occurred, as demonstrated by failure of endotoxin to cause maximal adrenal cortical stimulation when compared to intravenous ACTH. However, some animals which became tolerant to fever stimulating properties retained a normal maximal adrenal cortical response to endotoxin (Table III). It appears, therefore, that varying degrees of tolerance to adrenal cortical stimulating effect occur in the dog.

Fever tolerance in dogs in doses of 0.01 mg of endotoxin is very short lived and several days after cessation of endotoxin administration normal fever responsiveness occurs. Our experiments certainly establish that neither fever nor hypotension are prerequisites to adrenal cortical stimulating effects of endotoxin, a conclusion suggested by previous findings that adrenal cortical stimulation precedes appearance of fever in dogs given an initial dose of endotoxin(1). We do not conclude that partial adrenal cortical tolerance is due to an effect on adrenal, pituitary or elsewhere. That it is not the adrenal cortex seems likely because of normal responsiveness to exogenous ACTH. This is quite different from decreased responsivity to exogenous ACTH following larger quantities of endotoxin as previously described(1). It is quite possible that "partial tolerance" represents nothing more than decreased ACTH release by the pituitary. This possibility is at present being evaluated.

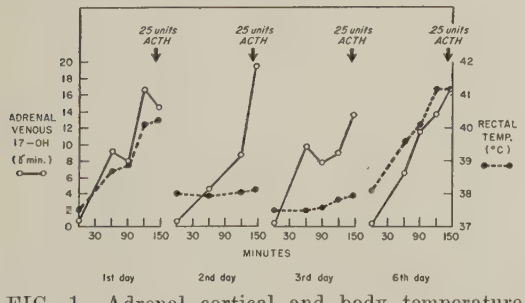

FIG. 1. Adrenal cortical and body temperature response following intrav. administration of 0.01 mg of endotoxin.

There is a precedent for the tolerance observed on second day of endotoxin administration. Bernhimer and Cantoni(6) found that mice became temporarily resistant to lethal effect of a streptococcus toxin after one injection of toxin, and that resistance was present 3-6 hours after initial dose and was gone by 40 hours. With regard to the mechanism of release of ACTH following endotoxin, spinal cord section does not prevent the response(7). Endotoxin is not a corticotrophin-like substance since it does not result in adrenal cortical stimulation in the hypophy-

sectomized animal. Accordingly it is proposed that endotoxin acts directly on the central nervous system to result in ACTH release. Experiments have been undertaken to determine site of action of endotoxin in the central nervous system for both adrenal cortical stimulating and fever-promoting properties.

Summary. Dogs given large doses of bacterial endotoxin do not become tolerant to either fever producing or adrenal cortical stimulating effects. Dogs receiving small doses of endotoxin readily become tolerant to the fever-producing effect, but either do not become tolerant at all or achieve only partial tolerance to adrenal cortical stimulating properties. The partial adrenal cortical tolerance observed following endotoxin administration probably represents decreased pituitary release of the ACTH rather than tolerance of adrenal cortex itself.

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A New Type of Lysis from Without. (24945)

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According to the enzyme hypothesis of phage action on susceptible bacterial cells(1-6), holes are made in bacteria cell wall, by phage enzyme following adsorption, and nucleic acids of the phage are injected through these holes into the cell. Within a few minutes after adsorption these holes are sealed (3), provided the damage to cell walls is not too great, as in lysis from without(1). One might therefore expect that if the phage enzyme acted on bacteria which were blocked in their ability to reseal even the minor "tears" in their walls, initial lysis would not be halted and the enzymatic action would disintegrate the wall completely. Such immediate lysis caused by T2r phage was observed by Heagy(7) in partially starved *Escherichia coli.* The present study shows that freezing and thawing of exponentially growing bacteria provides a shock which blocks repair of damage to bacterial wall, and that such thawed bacteria lyse immediately upon addition of the phage.

Methods. E. coli, strain B or B/r, was grown in broth or in a synthetic medium(8) until an optical density of 0.4-0.6 (at 540 mμ) was reached. This corresponded to a viable count of 2 to 4 x 10^8 cells/ml. The suspension was either directly frozen in the growth medium, using a CO_2-acetone bath, or it was washed by centrifugation and frozen in fresh medium. The bacterial suspensions were infected with various bacteriophages (T2r$^+$, T4D, T4Dr47, T3, or T7), either before freezing or after thawing. In the first case, phage was added to cells at a desired multiplicity of infection, the suspension incubated 3 min. at 37°, and frozen. In the second type of experiment, phage was added immediately upon thawing, *i.e.,* at moment of final disappearance of ice. The volume of added phage was 1/30 to 1/50 of the bacterial suspension, while multiplicity of infection varied between 1 and 15. For determination of onestep growth curves, unadsorbed phage was removed by centrifugation.

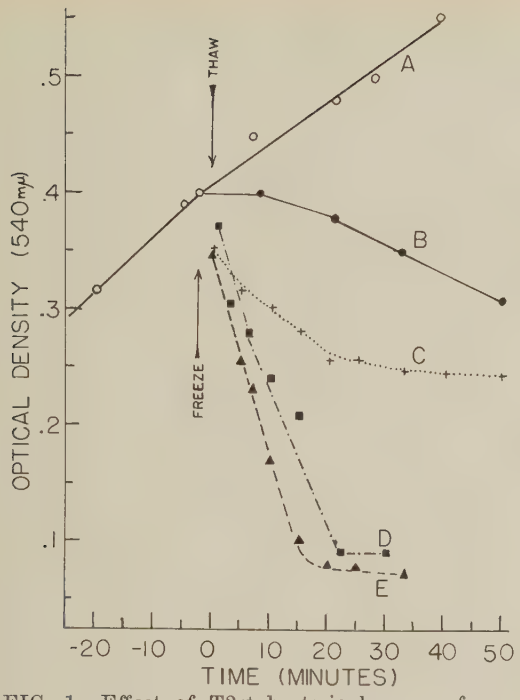

FIG. 1. Effect of T2r+ bacteriophage on frozen and thawed cells of *E. coli* B/r. Multiplicity of infection = .4. Curve (A) represents exponentially growing culture (at 37°) from which, at −3 min., 4 separate samples were derived. At this time phage was added to samples (B) and (E). Sample (B) was incubated undisturbed at 37°. Samples C, D, and E were immediately frozen at −80° and 2 min. later thawed. Phage was added to sample (D) immediately after thawing. (C) is the control lysis curve due to freezing and thawing alone.

Results. The fate of infected or non-infected frozen and thawed bacteria was followed by viable counts of bacteria (or of infective centers), as well as by measuring changes in optical density of suspension. One-step growth curves and single cell bursts were also determined in bacteria infected before freezing(8).

Freezing and thawing of a control suspension caused a loss of viability of 30 to 60% of bacteria. This loss was accompanied by 10 to 25% drop in optical density during first 10 to 20 min. after thawing at 37° (Fig. 1-C). Thawed bacteria did not resume growth for 1 to 2 hr. Freezing and thawing of infected bacteria caused a loss of about 80% of infective centers and an approximately equal drop in optical density (Fig. 1-E). Upon addition of phage after thawing of frozen bacterial suspension, lysis occurred in a manner and at rate similar to that in bacteria infected before freezing (Fig. 1-D). The concomitant lysis, as observed in photometer, reached 60 to 70% in nutrient broth at multiplicities of infection below 10, and within 20 min after thawing (*i.e.,* during latent period when no lysis should occur). In synthetic medium the lysis was less pronounced. Higher multiplicities of infection caused a more rapid and extensive lysis. In presence of 0.3 M sucrose and phage, the thawed bacteria form spheroplasts.

The effects of freezing of exponentially growing cells of *E. coli* are evident not only in decrease of viability of thawed suspension, partial lysis, and interruption of exponential growth of survivors for 1 to 2 hr, but also in their striking response to lytic phages. Frozen and thawed bacteria infected before freezing exhibit a longer than normal latent period at 37° and their burst size is smaller. The fact that such cells produce phage at all indicates that their synthetic mechanism is not damaged by freezing and thawing, although the ability of frozen, non-infected cells to divide is seriously impaired. As mentioned before, frozen bacteria to which T2r+, T4D and T7 phages were added after thawing lysed considerably, although multiplicity of infection was below that causing lysis from without. It has been shown by Puck and Lee (3) that in the second, enzymatic stage of adsorption of phages to bacteria, a local leakage of bacterial contents occurred and that this leakage was soon repaired. Similarly, Doermann(9) showed that after attachment of T2 phages to *E. coli,* a transient drop in optical density occurred. This was interpreted by him as a permeability change in bacterial cell walls infected by phage.

Discussion. In cell-wall preparations, lysis by phage enzymes (whole phages(5) or frozen and thawed phages(4)), as evidenced by appearance of labeled split products from the wall, was demonstrated by Barrington and Kozloff(5), Weidel and Primosigh(4), and Koch and Weidel(10). All these findings give strong support to the original Delbrück (1) enzyme hypothesis of phage action.

Our experiments fit this hypothesis. Because of dormancy induced by freezing shock,

the damage due to enzymatic dissolution of parts of cell wall is not repaired; bacteria swell and burst in a sort of sterile lysis, as evidenced by drop in optical density and concomitant loss of infective centers. As the kinetics of lysis of frozen and thawed bacteria are similar with different phages, one might assume with Weidel(6) that the substrate for them is common. Comparison of phage lysis with lysozyme lysis of frozen and thawed cells(11) indicates that phage enzyme is isodynamic with lysozyme. All this is consistent with an assumption that the shock of freezing and thawing causes tears or holes in the outer lipoproteid layer of the cell wall(6), thus admitting the enzyme or phage's tail to act on the inner, lipopolysaccharide layer. Integrity of enzyme substrate in this rigid layer is essential for maintenance of bacterial, rod-like, structure. When the substrate is lysed, and the repair is blocked by freezing shock, the inner wall layer disintegrates(2) and the bacteria either lyse, or form spheroplasts(12) if suspended in proper hypertonic medium. The formation of such spheroplasts by lysis from without (1000 T2 phage particles/bacterial cell) had been previously observed by Carey *et al.*(13). In the present study, spheroplasts were formed from frozen and thawed *E. coli* under the influence of only few phages.

Summary. Frozen and thawed cells of *E. coli* B and B/r lyse immediately upon addition of various T-bacteriophages (T2r$^+$, T4D, T4Dr47, T3 and T7), at multiplicities of 1-15. Lysis is complete before the end of latent period and is more rapid as higher multiplicities of infection. When phage is added to thawed bacteria in 0.3 M sucrose, spheroplasts are formed.

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Influence of Hair Growth Cycle on Cytochrome Oxidase and DPNH-Cytochrome C Reductase in Mouse Epidermis.* (24946)

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Although normal epidermis has been demonstrated to undergo pronounced morphological changes in relation to hair growth cycles, relatively little is known about corresponding alterations in chemical composition. Recently, histochemical studies have clearly demonstrated certain chemical changes in epidermal cells paralleling hair growth activity (1). It is well established that the chemical constitution of epidermis is significantly altered when malignant transformation occurs by chemical carcinogens(2). Incidence of skin tumors induced by chemical carcinogens also has been shown to vary considerably with hair growth activity(3). A proper interpretation of such findings depends on a complete knowledge of normal quantitative and qualitative alterations in epidermal chemical composition which accompany hair growth cycles. In the present study the activity of 2 enzymes, namely cytochrome oxidase

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and DPNH†-cytochrome c reductase, was determined in the epidermis at the 4th (anagen III), 12th (anagen VI) and 22nd (telogen) days of hair growth cycle(4). These stages represent maximal alterations in epidermal hypoplasia and hyperplasia or growth. Activity of cytochrome oxidase in actively proliferating hair follicles was also determined for comparison with normal hypoplastic and hyperplastic epidermis obtained at known stages of the hair growth cycle(5).

Methods. The hair was plucked by hand from adult Swiss albino mice with quiescent (telogen) hair. Skin samples were obtained from mice killed by cervical dislocation 4, 12 and 22 days following plucking. Epidermis was separated from the dermis at 50°C by procedure of Baumberger *et al.*(6) and dropped into several ml of water in 7 ml Ten Broeck homogenizer, immersed in crushed ice. The hair follicle preparation (hair bulbs, hair shafts and some hair) was obtained from skin of A and C_3H mice 10 to 14 days of age, by first dissecting the layer of skeletal muscle (panniculus carnosus) and a considerable amount of adipose layer from the skin (epidermis down). Hair follicles and associated material were scraped off with iris knife and placed into 2 ml distilled H_2O in 25 ml lusteroid centrifuge tube immersed in crushed ice. Hair follicle preparations were centrifuged at 13,000 rpm (International multispeed attachment and rotor No. 269) for 10 minutes at 0°C. The sedimented material was then homogenized by hand in several ml distilled H_2O. Epidermal preparations were also homogenized in similar fashion and aliquots of both homogenates were retained for nitrogen determinations by Kjeldahl procedure. Nitrogen appears to be as good a basis of reference as any other tissue constituent(2). The remainder of the homogenates were centrifuged at 1500 rpm (International horizontal rotor No. 241 for Model V, size 2 centrifuge) for 10 minutes at room temperature. Supernatant fractions thus obtained were kept cold and used immediately for enzyme assay. Epidermis removed from skin at various phases of hair growth cycle and follicular material were disrupted to about the same extent by homogenization. The reaction mixture for determination of cytochrome oxidase activity was as follows: A total volume of 3 ml contained 0.2 ml 0.17 M sodium phosphate buffer, pH 7.4; 0.4 ml 1.7×10^{-4} M reduced cytochrome c and 0.02 to 0.1 ml of sample. The volume was adjusted to 3 ml with H_2O. Cytochrome oxidase activity was determined according to the macro procedure of Cooperstein and Lazarow(7) by measuring change in optical density at 550 mμ every 15 seconds in a Beckman model DU spectrophotometer. For assay of DPNH-cytochrome c reductase activity, 3 ml of reaction mixture containing the following materials in concentrations indicated were used: 0.027 M nicotinamide; 2×10^{-4} M NaCN; 0.033 M phosphate buffer, pH 7.4; 1 mg cytochrome c; 300 μg DPNH; 0.03 to 0.1 ml sample and H_2O to make a final volume of 3 ml. Activity of DPNH-cytochrome c reductase was determined from rate of reduction of cytochrome c at 550 mμ every 15 seconds according to procedure of DeDuve *et al.*(8).

Results. Cytochrome oxidase activity of epidermis at day 4 of hair growth cycle was considerably greater than that found for epidermis removed at 12th and 22nd days of hair growth cycle, whether the results were expressed on the homogenate or supernatant nitro basis (Table I). The epidermis is hyperplastic at 4th day of hair growth cycle apparently due to increased mitotic activity(5,9, 10). The elevated level of cytochrome oxidase activity observed at this time thus may be associated with increase in cell division.

TABLE I. Cytochrome Oxidase Activity of Epidermis of Skin in Various Stages of the Hair Growth Cycle.*

Stage of hair growth	Day of cycle	Specific cytochrome oxidase activity† of Homogenate	Supernatant
Anagen III	4	2.2 ± .60	4.4 ± .80
"	12	.8 ± .25	1.7 ± .25
Telogen	22	1.1 ± .18	2.5 ± .22

* Results are avg with dev. from mean of 3 determinations using epidermis of 3 to 5 mice/analysis.

† △ log ferrocytochrome c/min./mg nitrogen.

† The following abbreviations are used: DPNH, reduced diphosphopyridine nucleotide; DNA, desoxyribonucleic acid; RNA, ribosenucleic acid.

TABLE II. DPNH-Cytochrome c Reductase Activity of Epidermis of Skin in Various Stages in the Hair Growth Cycle.*

Stage of hair growth	Day of cycle	Specific DPNH-cytochrome c reductase activity† of	
		Homogenate	Supernatant
Anagen III	4	1.2 ± .18	2.2 ± .50
"	12	.3 ± .05	.9 ± .20
Telogen	22	1.0 ± .15	2.1 ± .27

* Results are avg with dev. from mean of 3 determinations using epidermis of 3 to 5 mice/analysis.

† △ log ferrocytochrome c/min./mg nitrogen.

This assumption appears to be strengthened by the fact that cytochrome oxidase activity of growing hair follicles, which contain actively dividing epidermal cells(9) was much higher than that of epidermis. Average value of follicular material was 10.1 ± 3.87; even the lowest values were twice that of the epidermis removed on 4th day of hair growth cycle when cytochrome oxidase activity was a maximum (2.2 ± 0.6). Histological examination of follicular preparations showed no evidence of skeletal muscle which has a high cytochrome oxidase activity(11,12).

DPNH-cytochrome c reductase activity of epidermis on 12th day of hair growth cycle was significantly less than the activity of epidermis on 4th or 22nd days (Table II). At 12th day of hair growth cycle the epidermis is thinner than at any other stage of the cycle (5). No values were obtained for hair follicle preparation. Thus, both cytochrome oxidase and DPNH-cytochrome c reductase activities within epidermis appear correlated with the mitotic status of its constituent cells.

Griesemer has shown that the succinic dehydrogenase DNA† and cytochrome oxidase DNA† ratios of 4th day rat epidermis was about 30% higher than that of the epidermis of 0 day of hair growth cycle(13). However, no significant difference was found in activity of these enzymes on 0 and 4th day of cycle when enzyme activities were expressed on dry weight or RNA† basis.

Summary. 1. Cytochrome oxidase activity of epidermis of mice on 4th day of hair growth cycle was appreciably higher than of epidermis of 12th and 22nd days of cycle. 2. Hair follicle preparations from very young mice had much greater cytochrome oxidase activity than did epidermis of 12th and 22nd days of cycle. 2. Hair follicle preparations from very young mice had much greater cytochrome oxidase activity than did epidermis regardless of stage of hair growth. 3. DPNH-cytochrome c reductase activity of epidermis of 12th day of hair growth cycle was significantly less than that of epidermis removed on 4th and 22nd days of cycle. 4. Activity of cytochrome oxidase appears associated to some extent with mitotic activity of tissues examined.

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Independence of Sedative and Analgetic Antagonizing Effects of Two Reserpine Esters.*† (24947)

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Schneider(1), Sigg and Schneider(2) and Gross (personal communication) demontrated that reserpine has the property of antagonizing the analgetic effects of morphine. The fact that some stimulants including amphetamine(3) and certain other amines(2) can potentiate morphine analgesia suggests that sedative or stimulant properties of a compound may determine in part whether that compound will antagonize or potentiate analgetics. Recently Barrett and Plummer(4) described 2 reserpine esters which may be useful tools in elucidating this point since one of these (SU-5171) possesses a degree of sedation comparable to reserpine with minimal hypotensive effects while the other (syrosingopine) produces only about 1/20th the sedative effects of reserpine but yet retains hypotensive activity.

Materials and methods. Prolongation of reaction time (tail flick) to a constant intensity heat stimulus, according to the method of D'Amour-Smith(5) was used as criteria of analgesia. Mice (male CF-1, 19-21 g) were housed individually in stainless steel containers with only their tails protruding. Racks were used to hold 10 cages at a time in a line and were provided with shallow grooves into which the animal's tail could be laid. This rack was then placed on a flat surface which allowed it to be moved back and forth for alignment with the focal point of heat source as mounted above. This source consists of a DC powered end view 100 watt concentrated-arc bulb (Zenith) mounted vertically in an appropriate housing. Light from this source was then focused to a beam of approximately 2 mm diameter and adjusted to elicit a control reaction time of about 4 sec. A foot switch operated both a shutter blade attached to rotary solenoid (G. H. Leland, Inc., Dayton, Ohio) and a precision timer with a complete sweep of 10 sec. (Standard Electric Timer Co., Springfield, Mass.). Because of the high intensity of the emitted light, a double polaroid shield was placed between operator and light path and was rotated to the dark position when the light was on. Although this dampened the light intensity considerably, it still allowed the operator a full view of animal's reaction. The AD_{50} of morphine sulfate either alone or in combination with other agents was defined as that dose of morphine producing significant analgesia in half of the animals and was determined as follows: Control reaction time (duration of application of heat necessary to elicit a tail flick) was measured in duplicate in each of 10 mice before and every 15 minutes for 2 hours after morphine administration. Significant analgesia was said to occur in any animal when its duplicate reaction time exceeded the control mean of the 10 animals plus 2.3 (S.D.) at any time during the 2 hour period. AD_{50} values were calculated according to the graphic method of Miller and Tainter(6) using a minimum of 10 animals at each of at least 3 doses falling between probits 3.75 and 6.25. All drugs were administered subcutaneously. Reserpine (lyophilized Serpasil phosphate, CIBA), 2.5 mg/kg, syrosingopine, the carbethoxy syringate ester of methyl reserpate, 2.5 mg/kg and SU-5171 (methyl-18-0(dimethylamino benzoyl) reserpate), 2.5 mg/kg were administered 2 hours before administration of morphine. Both syrosingopine and SU-5171 were dissolved in N, N′-dimethylacetamide to give an injection volume of .04 ml/20 g of body weight. Methylphenidate was dissolved in water and administered in doses of 5 mg/kg at the same time as the morphine. Control studies were performed in which reserpine, syrosingopine, SU-5171, methylphenidate and N,N′-dimethylacetamide were given

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TABLE I. Effects of Reserpine, Two Reserpine Esters, Methylphenidate and Solvent on Analgesia.*

Compound	Control ± S.E., 95% confidence limits	Reaction time in sec. Post-inj. 15′	45′	90′	120′
Reserpine	4.11 ± .08 3.92–4.30	3.44‡	3.61‡	3.49‡	3.52‡
Syrosingopine	3.99 ± .08 3.82–4.16	3.95	3.87	4.44	4.43
SU-5171	4.01 ± .09 3.81–4.21	4.20	4.67	4.07	4.14
Methylphenidate	3.99 ± .06 3.85–4.13	4.72	4.31	3.83	4.18
ADMA†	4.06 ± .12 3.80–4.32	4.13	3.53	3.69	3.85

* Reserpine (lyophilized Serpasil phosphate, CIBA), 2.5 mg/kg (dissolved in water), syrosingopine, 2.5 mg/kg (dissolved in ADMA), and Su-5171, 2.5 mg/kg (dissolved in ADMA) as well as solvent control given in volumes of .04 ml/20 g body wt. All of above compounds administered 2 hr before post-inj. analgetic determinations. Methylphenidate dissolved in water and administered in doses of 5 mg/kg immediately before analgetic determination.

† N, N′-dimethylacetamide.

‡ Indicates significant difference from control ($p < .05$).

alone in doses and time sequence listed above to determine if they alone produced significant alteration of reaction time.

Results. Control studies (Table I) reveal that reserpine alone produced a significant shortening of the reaction time ($p < .05$) for the 2 hour test period when the drug was administered 2 hours before testing. Syrosingopine, SU-5171, methylphenidate and N, N′-dimethylacetamide caused no alteration in reaction time when given alone.

Methylphenidate in doses of 5 mg/kg produced a very significant potentiation of the analgetic effects of morphine as indicated by the fact that when combined with methylphenidate, the dose morphine could be reduced by almost a factor of 3 to achieve the same analgetic activity as morphine alone.

TABLE II. Interaction of Methylphenidate, Reserpine and 2 Reserpine Esters on Morphine Analgesia.

Compound	Dose, mg/kg	AD_{50} ± S.E.	95% confidence limits
Morphine sulfate		2.3 ± .26	1.7 –2.8
Idem + methylphenidate	5.0	.87 ± .09	.69–1.05
Morphine sulfate 2 hr after:			
Reserpine	2.5	5.6 ± .30	5.0 –6.1
SU-5171	"	5.7 ± .13	5.5 –6.0
Syrosingopine	"	4.7 ± .67	3.4 –6.0
Morphine sulfate + methylphenidate 2 hr after:	5.0		
Reserpine	2.5	2.6 ± .31	2.0 –3.2
SU-5171	"	2.4 ± .44	1.5 –3.2
Syrosingopine	"	2.9 ± .35	2.2 –3.5

Reserpine, SU-5171 and syrosingopine administered in doses of 2.5 mg/kg 2 hours before testing all antagonized morphine analgesia to a very significant degree ($p < .05$). Prior administration of these compounds required that 2 to 2.5 times as much morphine was needed to effect the same degree of analgesia as compared to morphine alone. These compounds were equally potent in producing this antagonism since the AD_{50}'s for morphine in animals pretreated with these 3 compounds were not significantly different from one another ($p > .05$).

AD_{50} determinations following administration of methylphenidate (5 mg/kg) immediately before analgesic testing with morphine 2 hours after administration of reserpine, SU-5171 and syrosingopine (2.5 mg/kg) were not different ($p > .05$) from the AD_{50} for morphine alone.

Discussion. Significant reduction in reaction time caused by reserpine, about 18%, cannot explain the antagonism that this compound exerts against morphine analgesia which amounts to 250%. In addition, neither of the 2 esters caused any alteration in reaction time of themselves, yet both antagonized

morphine with the same order of potency as did reserpine. This shortening of reaction time may well be another manifestation of 'stimulant' effects of reserpine, reflected as a lowering of electroshock threshold(7) and prolongation of rhinencephalic seizures(8) and would suggest that neither SU-5171 nor syrosingopine have these properties to the degree that reserpine does.

That syrosingopine, with a potency of 1/20 that of reserpine in causing sedation, produces the same degree of antagonism to morphine analgesia, rules out the possibility that the sedative effect *per se* is a causal factor in producing this phenomenon.

Summary. The sedative properties of reserpine or esters or reserpine seems to bear no relationship to ability of these compounds to antagonize morphine analgesia since one such agent (syrosingopine) possesses 1/20 the sedative properties of reserpine yet is equally potent in its ability to antagonize morphine analgesia.

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Effect of Molybdenum on Fluoride Retention in the Rat.* (24948)

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Adler(1) has shown that 0.1 μg Mo/ml (as ammonium molybdenate) reduces the incidence of dental caries in the rat. His studies were prompted by the observation that 2 communities in Hungary had low dental caries rates and low fluoride levels, thus suggesting that low caries incidence was not the result of fluoride in water. Spectrographic analysis of these waters showed that molybdenum was present in excess of that found in similar communities in which the incidence of dental caries was higher. Since these data have considerable practical importance it was of interest to learn more about the relationship between skeletal retention of fluoride in presence of molybdenum.

Methods. 24 weanling Sprague-Dawley strain rats, bred and raised in our laboratory, were divided into 4 groups according to initial body weight. Group 1 received fluoride-low drinking water (F = 0.01 μg/ml) *ad lib.* Group 2 received 50 μg/ml Mo (as $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$) in drinking water. Group 3 received 50 μg/ml F (as NaF) in drinking water, and group 4 received Mo and F at same concentration as animals in Groups 2 and 3. All animals received the same fluoride low stock corn diet (F = 0.5 μg/g). Animals were housed individually in raised screen cages in temperature controlled room. After 30 days the animals were sacrified, the femur removed from each animal and the carcass prepared for fluoride analysis by methods previously described(2).

Results. The data obtained are in Table I. During the 30 day experimental period animals receiving fluoride-low drinking water and 50 ppm Mo gained on the average of 87 g. Those which received 50 ppm F gained less weight (70 g), but those receiving both Mo and F gained (98 g). No animals died during experimental period.

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TABLE I. Effect of Molybdenum on Fluoride Retention in the Rat.

Group	Mean wt gain (g)	Carcass fluoride		Femur fluoride	
		Total (μg)	Conc. (μg/g)	Total (μg)	Conc. (μg/g)
Control	87	145 ± 21	26.4± 3.1	6.37± 1.03	35.5± 5.9
50 ppm Mo	87	212 ± 12	39.9± 3.7	15.37± 1.48	88.3± 7.0
50 " F	70	8200 ± 550	1640 ±65	392 ±23	2340 ±165
50 " Mo + 50 ppm F	98	9820 ± 390	1830 ±66	464 ±24	2560 ±115

Values include stand. dev.

When the carcass was used to evaluate fluoride retention, 145 μg of F was present in control animals, while animals receiving 50 ppm Mo had 212 μg of F. When animals received 50 ppm F, 8200 μg of F was found in the carcass, and when both Mo and F was fed 9820 μg was found. Similar ratios are evident when the femur is used as index of skeletal fluoride retention.

Discussion. The presence of Mo seems definitely associated with increasing retention of fluoride in the rat. This is demonstrated by 2 types of evidence. (1) It increases retention by apparently increasing availability of both fluoride present, as a constituent of the diet and also that small fraction present in drinking water; and (2), it increases retention of that available fluoride added directly to drinking water as sodium fluoride. When total fluoride in the carcass is used to evaluate fluoride retention, a 32% increase is found when 50 ppm Mo is added to low-fluoride drinking water as compared to group receiving no Mo. When both Mo and F are added to drinking water there is approximately 17% more fluoride in the carcass than when only F is present. Both of these differences have probabilities in excess of 0.01. These data suggest that one of the functions of Mo is to increase the availability of the fluoride ion.

Conclusions. The presence of 50 ppm Mo as ammonium molybdenate is associated with significantly more fluoride retention in skeletal and carcass of the rat, than when Mo is absent from drinking water. These data suggest that Mo may act metabolically to increase availability of fluoride ion.

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Spatial Summation of Pain. (24949)

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An old textbook concept, probably largely based on Hardy's work(1) maintains that pain does not show spatial summation. Recently however, Green(2) has shown that pain summation occurs for small areas at pain threshold. The present investigation is an attempt to resolve this problem. The approach used is crude. However it allows a comparative evaluation over a wide range of experimental conditions, practically impossible to obtain with more refined technics. A constant heat stimulus was applied and the elapsed time determined until the subject reported the first pricking pain (threshold). Then the size of the stimulation area was changed and threshold time was again determined. This was done for 3 different sizes of stimulation area. The entire procedure was repeated with the subject reporting the maximum bearable pain (supra-threshold pain). All the above procedures were then repeated for 2 other levels of heat stimulation. The heat stimulus

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TABLE I. Effect of Intensity and Area of Stimulation (Contact Heat) on Threshold and Supra-Threshold Pain.

Stimulus, °C	Area 70 cm²			Area 14 cm²			Area 3 cm²			Mean		
	t(s)	T(°C)	T.D.*	t(s)	T(°C)	T.D.*	t(s)	T(°C)	T.D.*	t(s)	T(°C)	T.D.*
A. Threshold pain†												
51	2.6	45.1	.32	4.7	46.9	2.21	9.7	47.9	9.25	5.7	46.6	2.15
55	1.3	44.1	.08	2.3	46.4	.75	4.1	48.3	5.36	2.6	46.3	.79
60	.7	44.0	.04	1.3	46.2	.36	2.4	48.6	3.90	1.5	46.3	.45
Mean	1.5	44.4	.11	2.8	46.5	1.00	5.4	48.3	7.05	3.3	46.4	1.01
B. Supra-threshold pain†												
51	4.9	46.9	2.31	11.8	48.2	14.4	48.2	48.3	62.9	21.6	47.8	18.7
55	2.5	46.7	1.16	4.4	48.5	6.65	7.9	49.6	26.5	4.9	48.3	5.79
60	1.5	46.7	.61	2.9	49.2	3.26	4.0	50.4	23.9	2.8	48.8	5.25
Mean	3.0	46.8	1.31	6.4	48.6	10.4	20.0	49.4	57.8	9.8	48.3	12.8

* T.D. = Tissue damage in mΩ (see text).
† Each value represents mean of 24 experiments in 12 subjects.

was a metal container filled with hot water which was maintained at a constant temperature (60°, 55°, and 51°C). Under these conditions the outside of the container had a temperature of 57.6, 53.0, and 49.6°C, respectively. These temperatures were determined with a Stoll-Hardy radiometer(3) and were practically identical over the entire field of stimulation. Exposure area was varied by covering part of the container with heat-insulating material, leaving free an area of 70 cm^2, 14 cm^2, and 3 cm^2. The site of stimulation was the volar side of the forearm, which was held by the experimenter in firm contact with the heat source. Twelve students and faculty members served as subjects. Final temperature at the site of the pain receptor was calculated. For this purpose pain receptors were assumed to lie at an average depth of 200 μ below the skin surface. Using this calculated skin temperature, intensity of heat stimulation and time of exposure, temperature at the pain receptor was calculated using Henriques' formula(4). As pain may be more directly related to tissue injury than to time of exposure or final temperature, tissue damage was similarly calculated(4). However, the unit used for damage was one thousandth of Henriques' omega, or one millomega: $1\ m\Omega = 3.1 \times 10^{101} \times t \times e^{-75,000/T}\ t + 273$. One "millomega" corresponds to damage caused by application of 45°C for 8.64 seconds.

Experimental *results* are summarized in Table I. For statistical analysis each subject was used as his own control. All the differences of stimulation areas are significant ($p < 0.05$) except the temperature values for supra-threshold pain resulting from stimulus temperatures of 51° and 55°. The differences of mean values are highly significant ($p < 0.01$) except those for temperature values at supra-threshold pain, which are significant only for $p < 0.05$.

When the skin is exposed to constant heat energy, an immediate and rapid temperature increase is observed. Gradually this diminishes until a balanced state is reached where heat gain equals heat loss. Threshold pain occurs at an early part of this curve. Therefore differences produced by spatial summation will be more clearly defined if expressed in terms of tissue temperature. At supra-threshold pain the heating curve is flatter, and differences of stimulation area come out clearer when time is being measured. Intensity of stimulation has a similar effect, and modifies the effect obtained by change of pain intensity. The interaction of these factors explains largely the time and temperature variability of the data shown in Table I. The evidence for spatial summation of pain is considered clear cut, since it occurs at all intensities of stimulation used for 2 widely different pain intensities, and over the entire range of areas examined. From common life experience we know that a burn the size of a needle prick is hardly felt, while a burn of the same intensity covering a large area may cause intense pain. Therefore, spatial sum-

mation of pain is a matter of every day experience. The denial of spatial summation for pain is probably due to the false assumption that injury is the stimulus for pain. Undoubtedly severe injury can occur with very little or no pain. This difficulty was explained recently when it was shown that pain is not caused by injury as such, but by the tissue reaction to injury(5). Therefore, scientific evidence as well as common experience indicate that spatial summation is an inherent characteristic of pain sensation.

Summary. Spatial summation of pain was determined by applying contact heat to the volar side of the human forearm. Evidence indicates that summation of pain occurs for large fields as well as for small fields, for threshold pain as well as for supra-threshold pain, and for high intensity stimuli as well as for low intensity stimuli. Therefore pain summation is considered an inherent characteristic of pain sensation.

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Action of Polylysine on Some Ascites Tumors in Mice.* (24950)

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Synthetic polypeptides of lysine reduce infectivity of tobacco mosaic virus(1); inhibit multiplication of bacteriophage(2,3); and protect chick embryos for a period after inoculation with animal viruses such as mumps virus(4), infectious bronchitis, Newcastle disease viruses(5), and influenza B virus(6). The antibacterial activities of synthetic polylysines and copolymers containing lysine have been demonstrated by a number of investigators(7-11). Because of activity of polylysine against viruses and bacteria it seemed worthwhile to screen polylysine, along with other synthetic polypeptides, against various tumors in mice. This communication reports increased survival time of mice bearing Ehrlich ascites carcinoma and TA3 ascites tumors after treatment with synthetic lysine polypeptides, and describes some cytological effects of polylysine on tumor cells.

Materials and methods. The polypeptides were synthesized from amino acid N-carboxyanhydrides. Lysine polypeptides isolated and tested as their hydrochlorides were prepared as described by Becker and Stahmann(12) and Tsuyuki, Tsuyuki, and Stahmann(13). Average degree of polymerization(DP) was determined from α-amino nitrogen analyses. Random copolymers containing lysine in combination with valine or leucine were prepared by copolymerization of corresponding N-carboxyanhydrides. Polyglutamic acid was synthesized as described by Green and Stahmann(14); Protamine sulfate was obtained from the Eli Lilly Co. The above polymers were tested against tetraploid Ehrlich ascites carcinoma in female Swiss mice. However, only the poly-DL-lysine (DP = 240) was used in additional assays against diploid Ehrlich ascites carcinoma and mammary adenocarcinoma TA3 ascites tumor in female Swiss and male BAF1 mice, respectively. In screening procedures, young adult mice

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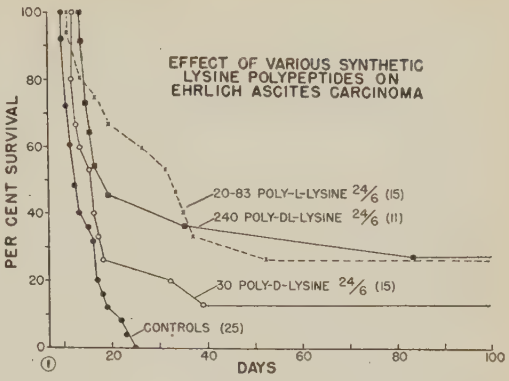
weighing 20 to 25 g were each transplanted intraperitoneally with 5 to 10 million tumor cells in isotonic saline as a 1 to 10 dilution of the packed cell volume. Treatment of mice was begun 24 hours after tumor transplant. Polymers were administered in isotonic saline so that each injection volume was 0.2 to 0.4 ml. Solutions were adjusted to pH 7.2 with sodium bicarbonate and then sterilized by passage through bacterial filter. Unless stated otherwise treatment schedule consisted of 2 intraperitoneal injections of the polypeptide solution daily for 6 days. An increased survival time of treated mice over control mice was used as the criterion of activity. The poly-DL-lysine (DP = 240) was also screened against the solid forms of Sarcoma 180 and Carcinoma 755 and against the ascites form of Leukemia L-1210. Screening procedures were essentially those outlined in Cancer Chemotherapy Report No. 1(15). Polypeptide solutions were prepared as before, and treatment schedule consisted of 2 injections daily for 7, 11, and 15 days for Sarcoma 180, Carcinoma 755, and Leukemia L-1210, respectively. Dosage in each case averaged 12 mg/kg for each injection. Criteria of effectiveness of treatment were the weights of excised solid tumors and survival time of mice with Leukemia L-1210. The polylysine used in cytological screening was poly-DL-lysine (DP = 240). The tumor used was the Ehrlich ascites carcinoma hyperdiploid Buffalo, chromosome mode (45-46) (16) maintained in this laboratory for 18 fluid transfer generations. Mice inoculated with 20 x 10^6 tumor cells were divided into 2 equal groups, a control group receiving injections of saline and a study group receiving polylysine 12 mg/kg/day i.p. This regimen was carried out over 6 days, daily weights of mice being used as estimate of tumor development. Mice were sacrificed on seventh day, and ascitic fluid withdrawn. Smears were made using Carnoy's fixative and stained by Feulgen's technic(17), with Toluidine Blue (18), or with Naphthol Yellow S(19) to investigate any changes in DNA, RNA and total protein of tumor cells. An aliquot of the ascites fluid was also treated after the method of Yoshida(20) to demonstrate chromosome



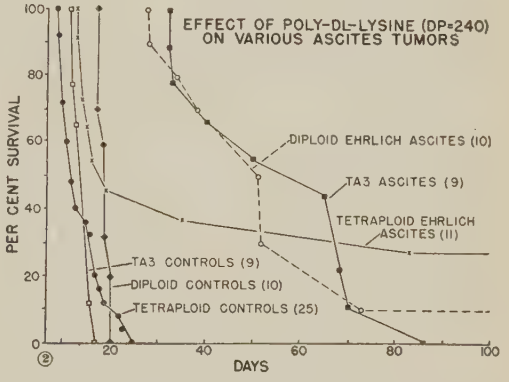


FIG. 1. Effect of various synthetic lysine polypeptides on survival of mice bearing Ehrlich ascites carcinoma.

FIG. 2. Effect of poly-DL-lysine (DP = 240) on survival of mice bearing various ascites tumors.

morphology. Cell volumes were measured by phase microscopy. Cells were maintained at 37°C unfixed, bathed in the ascites serum in hanging drop preparation. Diameters were measured by eyepiece micrometer and volumes calculated as spheres from this measurement. Total cellular nitrogen was estimated by Kjeldahl analysis with Nessler's reagent as described by Klein(21), cellular RNA and DNA by method of Schneider(22).

Results. In preliminary screening all of the polypeptides except the lysine polypeptides were ineffective against tetraploid Ehrlich ascites carcinoma. In initial experiments poly-L-lysine preparations of DP 20 and DP 83 inhibited growth of tetraploid Ehrlich ascites carcinoma. There was no significant difference between the 2 treated groups and an average of 27% of treated mice survived for 100 days, apparently free of tumor (Fig. 1). The untreated control mice all died between

TABLE I. Effect of Poly-DL-Lysine* on *In Vivo* Mitotic Cycle of Ehrlich Ascites Tumor Cells.

	% of cells in different phases of mitosis				
	Prophase	Metaphase	Anaphase	Telophase	Mitotic index
Control†	1.64	1.06	.19	.47	3.4 ± .6
Treated†	1.21	.23	.006	.10	1.4 ± .7

* DP = 240.

† 17,500 cells sampled.

10 and 25 days following transplantation. The effect of molecular size of the polypeptide and optical configuration of its lysine residues was studied. The results obtained with poly-DL-lysine (DP = 240) and with poly D-lysine (DP = 30) against the tetraploid Ehrlich ascites carcinoma are presented in Fig. 1. The data indicate that the DL isomer had about the same activity as the L isomer with average 27% survival at 100 days after transplantation without evidence of tumor formation. The poly-D-lysine (DP = 30) was the least effective with only 13% of mice surviving at 100 days with no apparent tumor.

Poly-DL-lysine (DP = 240) also showed activity against the diploid strain of Ehrlich ascites carcinoma; 10% of treated mice survived 100 days without any sign of tumor. Survival time of some mice bearing TA3 ascites tumor was extended to 86 days after transplantation (Fig. 2). It would appear that the poly-DL-lysine is most effective against tetraploid Ehrlich ascites carcinoma. This difference might be a function of tumor cell volume, for in the 1 to 10 dilution of the packed cell volume of tumors, a greater number of diploid and TA3 cells are transplanted. Generally, mice in the preceding experiments that died after first 3 to 4 weeks succumbed from solid tumors outside the peritoneal cavity rather than to recurrent ascites tumors. Subsequent development of solid tumors near site of implant were frequently observed.

The poly-DL-lysine (DP = 240) had no significant effect on growth of Sarcoma 180, of Carcinoma 755, nor on survival time of mice bearing Leukemia L-1210.

On removal of the ascitic fluid from treated and control mice, transplanted with Ehrlich ascites carcinoma, a noticeable difference in volume of ascites cells was seen, estimated as a packed cell volume from pooled samples. The treated mice showed half the volume of that of control mice. This finding is in agreement with daily weighings of the mice, controls showing usual increase in weight, whereas the treated group showed little or no weight increase. It should be noted that the dose of polylysine used in the cytological experiments was half of that used in survival time studies. The higher dose when used in preliminary studies did not allow multiplication of a sufficient number of cells for this study.

Analysis of mitotic activity of these tumor cells showed a marked decrease in later phases of the mitotic cycle, which would indicate that polylysine acts on tumor cells prior to these phases. From results tabulated in Table I it seems that this effect is most noticeable after prophase. There was little difference between control and treated groups at prophase. When the cell commences the mitotic cycle, the polylysine may inhibit at or previous to this stage and result in arrest at prophase. Overall, the mitotic index is reduced from 3.4 ± 0.6 for control value to 1.4 ± 0.7 for treated value.

In this examination, the frequency of multinucleated cells and mitotic abnormalities were also quantitated. The number of multinucleated cells showed a reduction from 1.27% in the control to 0.67% in treated animals.

Mitotic anomalies, although quantitatively small, appear significant rising to 0.33% in treated animals from 0.15% in the controls. The most frequent of these anomalies appeared at telophase; these may be described as (a) unsynchronized divisions to daughter cells, (b) daughter cells which, although in very late telophase, are still joined, (c) an occasional unipolar type of mitosis. In the control group the mitotic cycle showed a small percentage of mitotic anomalies characteristic of this tumor(23). Chemical analyses for nucleic acids and total proteins in treated and control cells showed no significant difference (Table II). Morphologically, resting cells of both groups were comparable, and there is

TABLE II. Cytological Analyses of Ehrlich Tumor Cells Treated *In Vivo* with Poly-DL-Lysine.*

	Cell vol, μ^3	DNA	RNA	Nitrogen
		$\mu\mu$g/cell		
Control	1150 ± 60	9.6	18.5	36.4
Treated	1240 ± 58	9.7	19.6	38.1

* Treated with 12 mg/kg/day of poly-DL-lysine (DP = 240) for 6 days.

no significant change in volume. Control cell volumes were 1150 μ^3 ± 66 μ^3 as compared to treated cell volumes of 1204 μ^3 ± 58 μ^3. Cytochemical preparations and morphological studies also indicated that there is no change in cellular volume, nucleic acids or proteins.

Discussion. Only the polypeptides of lysine had an inhibitory action on growth of ascites tumors. When the positive charge of the polylysine was dispersed as in leucine-lysine or valine-lysine copolymers, and in protamine sulfate, the antitumor action was lost. The ineffectiveness of polyglutamic acid against the tetraploid Ehrlich ascites carcinoma suggests that the high positive charge of polylysine may be necessary for activity. It has been reported that tumor cells develop an abnormally high negative charge when compared to homologous cells from which they are derived(24). The effectiveness of polymers with high positive charge, as polylysine, in inhibiting tumor development might involve an electrostatic interaction with the tumor cells. Such a specific interaction of polyethylene imine with Walker tumor cells has been demonstrated(25).

The antitumor action of lysine polypeptides apparently requires an intimate contact of polylysine with the ascites tumor cells within the peritoneal cavity. The refractoriness of solid tumors to polylysine might mean that the polypeptide does not reach the solid tumor in a concentration high enough to exert its inhibitory action. The high affinity of polylysine for red blood cells and tissue proteins could limit the systemic availability of polylysine. In the case of highly invasive leukemia L 1210, the tumor might be too highly metastatic to be significantly affected by the polylysine.

The observation that many treated mice either survived without development of ascites tumors or succumbed to solid tumors rather than to recurrent ascites tumors indicates a marked cytotoxic effect of polylysine against ascites tumor cells within the peritoneum.

Cytological studies of cells from the peritoneum showed marked tumor growth inhibition by polylysine as evidenced by decreased number of tumor cells in mitosis and a marked mitotic inhibition at the prophase level. The mitotic index in treated animals was about half that of controls and there is a marked mitotic inhibition at the prophase stage (Table I). Polylysine treatment does not change cell volume or nucleic acid or protein content (Table II), as is seen when Ehrlich ascites carcinoma is treated with 5-fluorouracil(26).

Summary. Synthetic polypeptides of lysine, of glutamic acid, of valine and lysine, of leucine and lysine, and commercial protamine sulfate were tested against tetraploid Ehrlich ascites carcinoma in Swiss mice. Only the synthetic polypeptides of lysine inhibited ascites tumor development. Up to 27% of mice treated with polylysine survived for 100 days apparently free from tumor. Mice that died after 3 to 4 weeks usually did so from metastatic solid tumors or solid tumors at site of implant. Polylysine also had a marked inhibitory activity against the diploid strain of Ehrlich ascites carcinoma and TA3 ascites carcinoma in Swiss and BAF1 mice, respectively. Survival time of some BAF1 mice was increased to 86 days, while 10% of Swiss mice survived 100 days with no sign of tumor. On cytological analysis, mitotic arrest at prophase was found. The nucleic acid and protein content/cell remained unchanged.

We wish to thank Dr. G. A. LePage for advice and assistance and Dr. B. E. Kline for assays on Sarcoma 180, Carcinoma 755 and Leukemia L-1210.

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Intratubular Fluid Movement in Dog Kidney during Stop Flow. (24951)

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The stop flow technic introduced by Malvin, Wilde and Sullivan(1,2,3) provides a convenient method for localizing reabsorptive and secretory activities in the mammalian kidney tubule. Briefly, the technic consists of clamping the ureter for a short period (*e.g.*, 8 minutes) during the steady state that arises from constant infusion of an osmotic diuretic. The intratubular fluid at the end of this period of ureteral occlusion differs from intratubular fluid during free flow because the former has had longer contact with tubular epithelium and, accordingly, its composition reflects reabsorptive and secretory activities to a greater extent. Immediately following the period of stop flow, serial samples of urine are collected and analyzed. The first sample is considered to be representative of fluid which is located most distal to the glomerulus. Each succeeding sample approximates fluid lying in more and more proximal positions. The arrival of fluid that entered the tubules after release of the occlusion is signaled by the presence of an indicator (*e.g.*, inulin) which was injected intravenously during the occlusion period. In interpreting the data so obtained, it has been assumed that the column of tubular fluid is stationary during the stop flow period. The present investigation explored the possibility that the column of tubular fluid does, indeed, move during the period of stop flow as a necessary consequence of water reabsorption. Thus, tubular fluid would be replaced in part by new filtrate throughout the stop flow period. This possibility has been mentioned before(2,4) but the magnitude of the effect may necessitate more attention than has previously been given. To demonstrate movement of tubular fluid during stop flow, we have repeated the technic described by Malvin, Wilde and Sullivan(2) with the modification that 2 or 3 indicators of

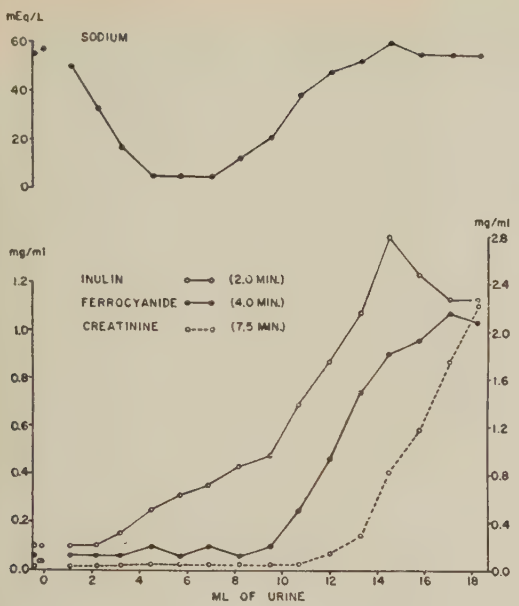

FIG. 1. Appearance of filtration indicators in stop flow samples. Figures in parentheses refer to time of inj. following ureteral occlusion. Left hand scale refers to ferrocyanide and creatinine; right hand scale refers to inulin.

glomerular filtration were injected at different times during the stop flow period. For example, in one experiment creatinine was injected 2 minutes after the ureter was clamped and sodium ferrocyanide was injected a few minutes later before the ureter was reopened. If movement of fluid along the tubule is negligible, creatinine and ferrocyanide should begin to appear in the same urine sample. On the other hand if there is appreciable fluid movement, creatinine should appear earlier than ferrocyanide. Further, the extent of the difference between the appearance of creatinine and ferrocyanide would be an indication of the extent of fluid movement during the period between injections.

Results. Fig. 1 illustrates the type of data we have obtained. In this experiment, 3 indicators were injected at different times during the 8 minute occlusion period. The first indicator, inulin, was injected 2 minutes after occlusion, sodium ferrocyanide was injected 4 minutes after occlusion and, finally, 7½ minutes after occlusion (*i.e.* ½ minute before reopening the ureter) creatinine was injected. Plasma samples taken from the femoral artery established that plasma concentration rose to its maximum at or before 15 seconds following each injection. Concentrations of inulin(5), creatinine(6), and ferrocyanide(7) were determined by conventional methods.

Inulin makes its appearance much earlier than ferrocyanide and ferrocyanide appears much earlier than creatinine. The Na curve (determined by flame photometry) has been included as a landmark. The region where Na concentration falls has been commonly interpreted as distal tubule. In these terms, our data indicates that new filtrate (*i.e.* plasma filtrate entering the tubules after the first 2 minutes of stop flow) has found its way into the distal regions of the tubules. Essentially similar results were obtained in 8 other experiments using various combinations of indicators, such as inulin and creatinine and inulin and ferrocyanide.

We interpret these movements of tubular fluid as being due primarily to water reabsorption by the tubules. It is true that a finite time is required for intratubular pressure to rise immediately after ureteral occlusion to the value existing in the glomerulus and during that time hydrodynamic flow would occur regardless of any water reabsorption. However, the experiments of Malvin, Wilde and Sullivan(2) indicate that ureteral pressure rises to a maximum within 1 to 1.5 minutes after occlusion. Since our first indicator was injected 2 minutes after urine flow was stopped, it would appear that this phenomenon plays an insignificant role in explaining our results.

Gross movement of tubular fluid during stop flow could be anticipated from U/P (urine concentration/plasma concentration) ratios of a continuously administered indicator. Our data as well as that of other investigators have shown that during a typical stop flow of 8 minutes duration, the intratubular U/P ratios vary from 2 to 6. Taking a mean U/P ratio of 4, for example, would indicate that total volume of water reabsorbate during the stop flow is roughly 3 times intratubular volume.

If new filtrate continuously enters the tubules during stop flow, then any change in plasma concentration occurring during or immediately following the stop flow period will

influence the composition of the collected samples. It follows that this factor must be accounted for if the method is to be used to calculate statistical distribution of nephron lengths and total volume of the nephron plus collecting system.

Finally, these results indicate that the actual localization of tubular activity by the stop flow method is perhaps less precise than realized. In particular, any quantitative conclusion that does not account for fluid movement and consequent introduction of new filtrate must be interpreted with caution.

Summary. The movement of intratubular fluid during stop flow has been investigated by injecting 2 or more filtration indicators at different intervals during period of ureteral occlusion. Appearance of these indicators in different samples collected upon release of the occlusion demonstrates that appreciable fluid movement takes place during stop flow.

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Differences between Antigenic Specificity of Nonsoluble Particles and Soluble Extracts Prepared from Brucellae by Disintegration.* (24952)

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Olitzki and Sulitzeanu(1,2,3) demonstrated that soluble antigens obtained by disintegration of Brucellae by sonic vibration or by a disintegrator reacted in agar gels with antisera and produced with them precipitation bands. On the other hand, Sulitzeanu(4) showed that nonsoluble particles resulting after disintegration of the majority of cells and after removing the rest of the intact bacteria by centrifugation, although unable to produce precipitation bands in agar gels, absorb agglutinins, while the soluble antigens do not possess this ability. Since in these experiments only *Brucella suis* was employed, the following experiments were carried out with *Br. suis, Br. abortus* and *Br. melitensis* to ascertain whether the antigens involved in these 2 different seroreactions are specific or not.

Methods. The antigens were prepared by the method of Olitzki and Sulitzeanu(3). From the 2 methods of immunization described by them the subcutaneous method with employment of an adjuvant was preferred. The bacteria were repeatedly disintegrated in a disintegrator (Mickle, Mill Works, Gomshall, Surrey) and remaining intact bacteria and cell walls were removed by centrifugation. For the agglutination test with intact bacteria acetone dried bacteria suspended in physiological saline were employed. 0.04 mg/ml of dried bacterial substance still gave a measurable opacity (O.D. 0.014) at 660 mμ and a visible agglutination. Cell wall antigens were prepared from disintegrated bacteria by centrifugation. At first the intact bacteria were removed by repeated centrifugation at 3400 rpm until no more sediment was produced. Then the supernatant was centrifuged 30 minutes at 9000 rpm. The absence of intact bacteria was verified by microscopical examination. The resuspended sediments gave opalescent suspensions, and

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TABLE I. Absorption of Precipitins from 1 ml of *Brucella melitensis* Antiserum Diluted 1:4.

Absorbing antigen prepared from	Absorbing quantity (mg/ml)	No. of bands appearing in agar gel with *Br. abortus*	*Br. suis*	*Br. melitensis*
Br. melitensis	2.4	0	0	0
	1.2	0	0	1
	.6	0	0	1
	.3	0	1	2
	.15	0	1	2
Br. suis	2.4	0	0	0
	1.2	0	0	1
	.6	0	0	1
	.3	0	1	2
	.15	1	1	2
Br. abortus	2.4	0	0	0
	1.2	0	0	1
	.6	0	0	1
	.3	0	1	3
	.15	1	1	3
Control, not absorbed		3	3	3

for agglutination tests higher optical densities of at least 0.074 were required. The best results were obtained at O.D. 0.124. The cell wall agglutination was different in its appearance from the agglutination of intact cells. Large flakes appeared and sedimented slowly in contrast to the granular, small flaking type of bacterial agglutination.

Results. Table I shows results of an absorption experiment with *Br. melitensis* and *Br. abortus* immune sera. By absorption with intact bacteria almost the same results were obtained as those obtained by absorption with cell walls. After addition of 25 mg of intact dried bacteria or dried cell walls to 5 ml of immune serum diluted 1:10 almost all agglutinins were removed when the homologous antigen was employed. However, when the heterologous antigen was employed, *e.g., Br. melitensis* in *Br. abortus* antiserum, then only the antibodies for the heterologous antigen were removed, and there still remained antibodies for the homologous antigen. Thus cell walls exhibit the antigenic scheme described by Miles and Wilson(5) for intact bacteria, according to which there is a prevalence of antigen M over antigen A in *Br. melitensis* and a prevalence of A over M in *Br. abortus*.

The result of absorption with moderate quantities of antigen must, therefore, be the production of a monospecific antiserum anti M for *Br. melitensis* and anti A for *Br. abortus*.

Completely different results were obtained in absorption experiments with bacterial extracts liberated from insoluble cellular components by centrifugation. These antigens were completely non-specific, since by employment of any of the 3 antigens in sufficient quantities all antibodies could be completely removed.

Table II shows that after absorption of *Br. melitensis* antiserum diluted 1:4 with minimal amounts of disintegrated bacterial substance as 0.6 mg/ml the majority of precipitating antibodies have already disappeared. When 2.4 mg/ml of the absorbing antigen were employed, this process was complete and no more precipitation bands appeared. The quantitative differences between these 2 reactions are evident. For incomplete absorption of heterologous agglutinins from 5 ml of se-

TABLE II. Absorption of Agglutinins by Cell Walls and Intact Bacteria.

Immune serum	Absorbing antigens		Reciprocals of agglutination titers with Intact bacteria *Br. melitensis*	Intact bacteria *Br. abortus*	Cell walls *Br. melitensis*	Cell walls *Br. abortus*
Br. melitensis	*Br. melitensis*	i.b.	20	10	20	0
	abortus	"	200	20	200	20
	melitensis	c.w.	20	20	20	0
	abortus	"	200	20	200	20
	Not absorbed		2,000	2,000	1,000	2,000
Br. abortus	*Br. melitensis*	i.b.	20	1,000	20	500
	abortus	"	10	0	20	20
	melitensis	c.w.	20	1,000	20	500
	abortus	"	0	50	20	20
	Not absorbed		1,000	2,000	500	1,000

i.b. = intact bacteria; c.w. = cell walls. 0 = agglutination negative at dilution 1:10.

rum diluted 1:10, 25 mg of total bacteria or cell walls were required, which corresponds to 50 mg of absorbing substance for 1 ml of undiluted serum. For complete absorption of all precipitins from 1 ml of a serum diluted 1:4 2.4 ml of disintegrated bacterial substance were required, which corresponds to 9.6 mg/1 ml of undiluted serum.

Summary. Bacterial agglutinins were removed partly by intact bacteria or cell walls from brucella antisera and by absorption with heterologous antigens monospecific antisera were produced. Cell wall agglutination parallels almost completely the agglutination of intact bacteria, when cell wall suspensions of high density were employed. Precipitins for soluble bacterial antigens demonstrable by agar gel precipitation technic were nonspecific and were removed by relatively small amounts of absorbing antigens.

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Indirect Effect of X-Irradiation on I^{131} Uptake in Chick Embryos.* (24953)

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Changes found in metabolism of I^{131} by thyroid in various animal species following total or partial body exposures to ionizing radiations are attributed to possible systemic response to exposures(1-6), but the evidence offered in support of this idea is circumstantial in nature. The following data afford direct evidence that changes in I^{131} uptake by thyroid in chick embryos are indirectly mediated by a substance found in peripheral circulation shortly after exposure of embryos to ionizing radiations.

Materials and methods. Fertilized eggs of White Plymouth Rock strain were obtained from commercial supplier. All injections were made into the air sac. Exposures to 600 r of x-rays were made approximately one hour after injections. Factors were 250 kv, 15 ma, Al parabolic plus 0.5 mm Cu with a HVL of 2.15 mm Cu, 25 cm Target-skin distance, and output 20.3 r/min. Approximately 24 hours after exposures and/or injections, the neck sections containing the thyroid were dissected out and counted for one minute in scintillation type well counter. The following experimental procedures were done. In Exp. 1, 12 or 15-day-old eggs were injected with 5 μc or 2.5 μc of I^{131}, respectively, about one hour before exposure to 600 r of x-rays. Controls received I^{131} only. In Exp. 2, 5 μc of I^{131} and 0.1 ml of pooled sera obtained from 13½ and 14-day-old embryos that had been exposed to 600 r of x-rays on 13th day were injected into 14-day-old eggs. Controls were injected with 5 μc of I^{131} and 0.1 ml of normal saline. In Exp. 3, 2.5 μc of I^{131} and 0.1 ml of pooled sera obtained from 15½-day-old embryos that had been exposed to 600 r of x-rays at 15 days of age were injected into 15-day-old eggs. Controls were injected with 2.5 μc of I^{131} and 0.1 ml of pooled sera obtained from untreated 15½-day-old embryos.

Results. The data shown in Table I indicate that not only does exposure of embryos to 600 r of x-rays result in increased uptake of I^{131} by the thyroid, but that pooled sera obtained from embryos exposed to 600 r of x-rays and injected into untreated eggs results

* This paper is based on work performed under contract with U. S. Atomic Energy Comm. at Univ. of Rochester Atomic Energy Project, Rochester, N. Y.

TABLE I. Effect of X-Irradiation and Pooled Sera from X-Irradiated Embryos on Uptake of I^{131}.

Exp. groups	Age in days	Sample size	Counts/min. (mean value)	p*
Control I	12–13	66	7,414 ± 591†	
I (600 r)	"	106	10,195 ± 438	.005
Control I	15–16	37	17,278 ± 1205	
I (600 r)	"	45	21,380 ± 1512	.05
Control II	14–15	9	17,903 ± 3879	
II (pooled sera)	"	15	31,417 ± 3848	.05
Control III	15–16	16	16,125 ± 1528	
III (pooled sera)	"	10	23,701 ± 2053	.01

* Significant level for difference between means of treated and control calculated by means of Student's "t" test.

† Stand. error of mean.

in a similar increase in thyroidal uptake of I^{131}.

Conclusion. This finding gives direct evidence that the change in uptake of I^{131} by the thyroid following exposure of chick embryos to x-rays is indirectly mediated *via* a substance found in plasma after exposure.

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Specificity of Anamnestic Response in Chickens.* (24954)

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The phenomenon of anamnesis, as originally recognized by Solomonsen and Madsen (1) in 1896, referred to the secondary response to diphtheria toxin of animals previously immunized with the same antigen. However, the term "Anamnestische Serum Reaktion" was coined to describe observations of Conradi and Bieling(2), in 1916, that soldiers previously vaccinated with typhoid vaccine had a sharp rise in typhoid antibody titer during the course of unrelated febrile diseases. Since these early observations numerous workers have reported increases in antibodies to a primary antigen following secondary stimulation with unrelated antigen [*Cf.* reviews by Wilson(3) and Freund(4)]. Others, however, have been unable to demonstrate such secondary non-specific responses(3-5). Recently, Dixon and Maurer(5), using quantitative technics, have shown that rabbits previously immunized with one antigen accelerate and magnify the immune response to primary injection of a heterologous antigen, only if the 2 antigens are related. Furthermore, the secondary non-specific responses occurred only if the primary and secondary antigens were related. In attempts to define the specificity of the anamnestic response, the common fowl has been but little employed as antibody producer, despite the fact that the fowl has the advantage of reacting rapidly to single small injections of a variety of antigenic substances and producing antibodies in a relatively higher titer, reaching a peak in 7 to 9 days(6-8). It

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TABLE I. Circulating Antibodies following Primary and Secondary Injections.

Group	Primary antigen*	No. of chickens	1st heterologous inj. Antigen	1st heterologous inj. Response†	2nd heterologous inj. Antigen	2nd heterologous inj. Response
I	HGG	18	Crys-BSA	BSA		
II	Crys-BSA	21	HGG	HGG		
III	Powd-BSA	13	Powd-BSA	BSA & HGG		
IV	"	14	HGG	"		
V	"	7	"	"	Hemocy.	Hemocy.
VI	"	1	Hemocy.	Hemocy.		
VII	Crys-BSA & HGG	6	HGG	HGG		
VIII	"	1	Powd-BSA	BSA & HGG		
IX	HGG	10	"	BSA		
X	"	11	HGG	HGG		
XI	"	1	"	"	PSG	PSG
XII	HA	1	Thyrogl.	Thyrogl.	"	"
XIII	"	1	HA	HA	"	"
XIV	"	1	Thyrogl.	Thyrogl.	Hemocy.	Hemocy.
XV	Hemocy.	2	Crys-BSA	BSA	Crys-BSA	BSA
XVI	PSG	2	Hemocy.	Hemocy.		
XVII	Thyrogl.	10	Crys-BSA	BSA	Hemocy.	Hemocy.
XVIII	"	1	HGG	HGG		
XIX	"	1	Thyrogl.	Thyrogl.	PSG	PSG

* In all cases animals produced detectable antibodies to primary antigen(s) at 8 days following inj.

† No attempt was made to differentiate degree of response: thyroglobulin injections resulted in weakly flocculating reactions; all others reacted quite strongly.

would seem likely, therefore, that immune responses of the fowl might be profitably employed in further elucidating this problem of specificity of the anamnestic response.

Materials and methods. One hundred and twenty-two male New Hampshire Red and Arbor Acre White Rock cockerels, ranging in age from 5 to 8 months, were employed. Seven relatively pure soluble proteins were used as antigens: Human gamma globulin (HGG), Cutter Labs, Human serum albumin (HSA), Cutter Labs, Crystalline bovine serum albumin (Crys-BSA), Armour and Co., Powdered bovine serum albumin (Powd-BSA), Pentex, Inc., Hemocyanin (Hemocy.), from Keyhole Limpet,† Thyroglobulin (Thyrogl.), Warner-Hudnut, Pumpkinseed globulin (PSG), Difco Labs. A micro-diffusion agar technic(9) was used to test the specificity of hyperimmune sera against BSA and HGG antigens. The powdered BSA was shown to contain trace amounts of globulin and HGG contained trace amounts of albumin. The animals were divided into 19 groups and treated as described in Table I. In each case the primary injection consisted of a single intravenous injection of 40 mg of antigen/kilo body weight. The animals were bled 8 days following injection and the presence of antibody determined by flocculation tests. The sera of Groups I and II were also analyzed for antibody Nitrogen (AbN) by quantitative precipitin technic previously described(8). Four to 8 weeks later the chickens were injected with either an homologous or heterologous antigen and bled at various intervals following this second injection. In addition, several groups of birds received a third injection with a heterologous antigen at intervals of 4 to 8 weeks following secondary injection. Each injection was preceded by bleeding, which was tested for presence of antibodies to previously injected antigen(s).

Results. As shown in Table I, secondary injections of one or more antigens acted only as primary stimuli to the antigens used, and did not elicit antibodies to previously injected antigens except in those cases where a definite relationship between primary and secondary antigens was shown. A secondary injection of HGG following disappearance of antibodies to a primary injection of Powd-BSA caused reappearance of anti-BSA antibodies (Groups III, IV, V). It was also found that a secondary injection of Powd-BSA (Group VIII) following a primary combined injection of Crys-

† Generously supplied by Dr. D. H. Campbell, California Inst. of Technol.

BSA and HGG recalled antibodies to HGG.

Quantitative studies were carried out on Groups I and II to analyze the effect of previous immunization with one antigen on the primary antibody response of a heterologous antigen. To this end Group I was given HGG as primary antigen and Crys-BSA as secondary antigen. At 8 days following primary injection, all animals produced an average AbN titer of 493 μg/ml of undiluted serum (μg/ml serum). Bleedings made 5 weeks after this primary injection showed no detectable antibodies to HGG in the circulating system. Furthermore, the secondary injection of BSA did not elicit detectable renewed response to the primary antigen when tested at 4 and 8 days following heterologous injection. On the other hand, average AbN value of 582 μg/ml serum for the response to BSA approximated that of 479 μg following primary injection of BSA in Group II.

Group II, initially given a single intravenous injection of Crys-BSA, responded with an average AbN titer of 479 μg/ml serum at 8 days following primary injection. There were no detectable anti-BSA antibodies in the circulating system when these animals were tested at 4 to 5 weeks after primary injection. At this time they were given a secondary injection of HGG. When tested for presence of antibodies to BSA and HGG at 4, 5, and 8 days following secondary injection, this group showed no antibodies to the primary antigen. Furthermore, the average AbN value of 485 μg is approximately equivalent to average titer of 493 μg obtained following primary injection of HGG in Group I. Thus, the primary response to HGG was unaffected by previous immunization of the same chicken with BSA.

Groups I, II, VII, X, and XI which received HGG either as a primary or secondary injection might be expected to contain heterologous antibodies following secondary injection. However, they did not. This could conceivably be attributed to the exceedingly small amount of contamination of albumin in the HGG.

Discussion. The confusion regarding specificity of the anamnestic response has largely been due to use of such complex antigens as serum proteins(10), polysaccharides(11), erythrocytes(12), and bacteria(13). Furthermore, little attention was paid to the relationship between primary and secondary antigens as the use of such closely related substances as sheep and horse sera by Hektoen(14), and typhoid, paratyphoid, and dysentery bacilli by Tsukahara(15). In many cases the interval between primary and secondary injection was arbitrary and the animals were not tested for presence of antibody at time of secondary injection. Furthermore, most early workers used the rabbit as their experimental animal (3) which normally requires a series of injections over relatively long periods of time to induce significant antibody responses. Although multiple injections are usually effective in enhancing antibody titers, numerous studies(16,17) have shown that such procedures tend to reduce the specificity of antisera produced against both soluble and particulate antigens. The present study employed the chicken which reacts rapidly to single small injections of antigen to produce antibodies of relatively high titer. The antigens used were all relatively pure soluble proteins. All animals were tested for presence of antibody previous to secondary antigenic stimulation.

In no experiments was it possible to demonstrate a non-specific anamnestic response due to stimulation with one or more secondary antigens if this material showed no cross reactivity with the primary antigen. The reappearance of anti-BSA antibodies following a secondary injection of HGG subsequent to a primary stimulation with Powd-BSA was conceivably elicited by the albumin contaminant in the HGG. In the reciprocal experiment the recall of antibodies to HGG following injection of Powd-BSA subsequent to a primary injection with Crys-BSA and HGG could conceivably have been elicited by the gamma globulin contaminant in Powd-BSA. In either case, the contaminant in the second injection might reasonably be regarded as having acted as a secondary stimulus to the cross reacting component in the primary antigen. Furthermore, the quantitative antibody response to the secondary antigen was unaffected by previous stimulation of antibody mechanism with primary antigen as evidenced by comparison

with previously uninjected control groups.

Summary. 1) The effects of secondary injections of various soluble protein antigens, subsequent to disappearance from the circulating system of antibodies to a primary antigen, are described. 2) Secondary antigen injections do not elicit antibody responses to previously injected antigens, if the antigens are unrelated. 3) Quantitative levels of antibody response to secondary antigens are apparently unaffected by previous antigenic stimulations of the animal, if the antigens are unrelated.

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Nychthemeral Variations in Plasma Free Corticosteroid Levels of the Rat.* (24955)

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Measurements of "resting levels" of plasma corticosteroids in a series of experiments in the rat relating to CRF activity *in vivo*(1) suggested a diurnal rhythm in this index of adrenocortical function. If (diurnal) variations were as large as we suspected, it was important to obtain an accurate level/time relationship, and an exact estimate of extremes of these variations. No report on similar investigations has been found in the literature, owing probably to recent availability of methods to measure plasma corticosterone levels in the rat.

Materials and methods. A group of 100 animals (rats, males, Holtzman Farms) were housed in our animal quarters (illuminated with daylight only) 40 days prior to start of experiment. On day of experiment (Jan. 31 to Feb. 1), one animal at a time was taken from a different cage and was decapitated within 20 seconds. Sacrifice of animals (B.W. 200-230 g) was done outside animal quarters, at 12 noon, 4 p.m., 8 p.m., 12 midnight, 4 a.m., and 12 noon. Blood was collected in 50 ml beakers containing 0.01 ml of heparin solution 1/1000, and samples taken for hematological studies. Total white cell counts were done with routine laboratory methods. Air-dried blood specimens were stained with Wright-Giemsa solution, and 500 leukocytes identified for each differential count. The absolute number of eosinophils, neutrophils, lymphocytes, and monocytes/mm^3 of blood was calculated from total white cell and differential counts. Calculated eosinophil values determined in this manner are comparable to those obtained by direct counting methods (Higgins, G.M., personal communication).

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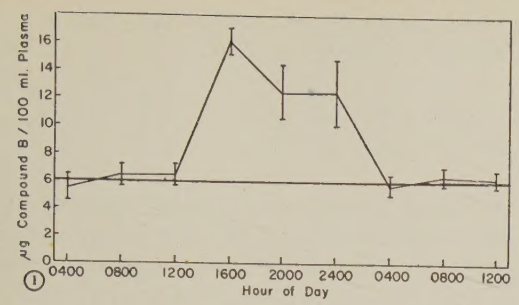

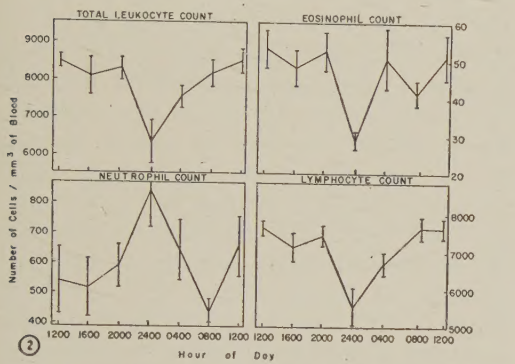

FIG. 1. Resting levels of plasma free corticosteroids at various times of day.

FIG. 2. White cell counts in peripheral plasma at various times of day.

Measurements of plasma-free corticosterone levels were done on 0.5 ml plasma samples as described by Guillemin *et al.*(2,3). Results are expressed in μg-corticosterone/100 ml plasma.

Results. Fig. 1 shows that striking variations occurred in concentrations of plasma free corticosteroids. With animals of this strain, body weight range, at this time of year and with schedule followed here for blood sampling, the highest concentration of plasma corticosteroids was observed at 4 p.m. with an elevated level persisting till midnight. In the rat, allegedly a "nocturnal" species, the nychthemeral rhythm appears to have an image inverted from the model known to exist in the human. Confirming other reports(4,5,6), variations were also noted in total white cell count (Fig. 2). Eosinopenia, lymphopenia, and neutrophilia at midnight are of interest in view of similar well documented responses of the peripheral blood elements following administration of ACTH or adrenal corticosteroids or exposure to stressful stimuli. The peak of leucopenia is not "in phase" with that of corticosteroid concentration, a fact best explained by the well known causative relationships between the 2 phenomena.

The literature on 24-hour periodicity of biological phenomena and particularly of pituitary-adrenal function is considerable (see review(7) by Halberg for references to 1953).† More recently evidence has been presented involving various structures of the limbic system in integration of cyclicity of the adrenocorticotrophic function of anterior pituitary (8). These studies were done on monkeys. The data reported here indicate that the rat may be used for similar studies since sensitive methods are available for measurement of plasma corticosteroids in this species. These data also add another reason to be considered in the care necessary(2) to obtain reliable "resting levels" of pituitary adrenal function in the rat.

Summary. Nychthemeral variations in levels of plasma free corticosteroids in the rat have been observed, with highest concentrations of steroid between 4 p.m. and midnight. The magnitude of these cyclic variations is such that they will have to be considered in measurement of "resting levels" of plasma corticosteroids in this species.

† Since this paper was submitted for publication, Halberg *et al.*(9) reported studies in mouse similar to those described here and with similar conclusions.

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Mucin-Like Enhancement of Microbial Virulence for Mice by Bile Salts and Certain Surfactants. (24956)

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While attempting to elucidate the mechanism by which bile salts exert their variable effect upon *in vitro* activity of a number of antimicrobial agents(1,2), it was observed that simultaneous intraperitoneal injection into mice of 1% mixture of equal parts of sodium taurocholate and sodium glycocholate and an inoculum of a strain of *Staphylococcus aureus* resulted in death of nearly all animals, whereas administration of same concentration of each alone *via* same route, proved innocuous. In light of this observation it was believed of interest to ascertain whether virulence of other microbial species known to be increased with the aid of gastric mucin(3) could be similarly enhanced by bile salts mixture and by other surfactants as well, both anionic and non-ionic. Because of their marked antimicrobial action even in high dilution, cationic surfactants could not be tested in this regard.

Materials and methods. Anionic compounds arbitrarily selected for investigation were di isopropyl naphthalene sodium sulfonate (Naccosol A) and di hexyl sodium sulfosuccinate (Aerosol MA); non-ionic compounds were alkyl aryl polyether alcohol (Triton X-100) and polyoxyethylene sorbitan monooleate (Tween 80). Indicated concentrations of the above, with exception of Naccosol A, and of bile salts were dissolved in distilled water and sterilized by autoclaving at 15 lb for 15 minutes. Naccosol A was added aseptically to sterile distilled water as heat sterilization causes this solution to become turbid, and then controlled for sterility before use. Five % gastric mucin (Type 1701-Wilson) was prepared according to Miller(4). A freshly isolated sample strain of each microbial species tested was grown in appropriate media for 18 hours at 37°C and then centrifuged at 2500 rpm for 45 minutes. Supernates were then replaced by equal amount of physiological saline to remove potentially lethal toxin formed during growth. After determining maximum non-lethal dilution of each strain and of each of the 4 surface active agents investigated upon test mice, 0.25 ml of appropriate dilution of each strain (Table I), was injected intraperitoneally into each of 7 groups of 10 albino male Swiss mice weighing between 15-20 g immediately after they had received by same route 0.5 ml of: 1) physiological saline; 2) 5% hog gastric mucin; 3) 1% bile salts mixture containing equal parts of sodium taurocholate and sodium glycocholate; maximum tolerated concentration of different surfactants, namely: 4) 1% Naccosol A; 5) 0.75% Aerosol MA; 6) 0.5% Triton X-100; and 7) 1% Tween 80. Deaths occurring in each group within 48 hours were noted. In addition, heart blood cultures were placed on media selective for the injected strain immediately after expiration if death occurred during the day and early morning, if the animals died during the night and positive results tabulated. Hydrogen ion concentration was measured electrometrically. Relative viscosity was computed by determining efflux time from calibrated tube of uniform bore at constant temperature as compared to distilled water. Surface tension was measured with Cenco Du Noüy tensiometer at 25°C.

Results. Number of fatal bacteremias produced in each group with the aid of different adjuvants investigated is noted in Table I. Except for Tween 80, all proved capable of enhancing virulence of a wide variety of microorganisms, converting ordinarily innocuous infections in animals, into a high proportion of fatalities associated with septicemia. 1% bile salts failed to raise virulence of our *Neisseria meningitidis* and *Salmonella choleraesuis* strains, but increasing the concentration to 2% (which was well tolerated), did result in mortality of 4/10 and 6/10 respectively. *Candida albicans* virulence was also increased by adjuvants although heart blood cultures were

TABLE I. Mouse Mortality/10 and Positive Post-Mortem Heart Blood Cultures Elicited by a Variety of Microorganisms with Different Surface-Active Agents as Compared to Physiological Saline and 5% Gastric Mucin.

Organism	Inoculum dilution, 18 hr culture (.25 ml)	Phys. saline	Gastric mucin 5%	Bile salts mixture 1%	Naccosol A 1% (.5 ml)	Aerosol MA .75%	Triton X-100 .5%	Tween 80 1%
Candida albicans	Undil.	0	4 (0)*	8 (4)	8 (0)	6 (0)	10 (0)	0
Hemophilus influenzae	10–1	0	10 (10)	2 (0)	2 (2)	8 (8)	8 (4)	0
Neisseria meningitidis	Undil.	0	10 ”	0 †	10 (6)	10 ”	5 (1)	0
Staphylococcus aureus	”	0	9 (7)	10 (6)	8 ”	9 (5)	10 (3)	1 (0)
Aerobacter aerogenes	10–1	0	9 (9)	6 ”	7 (7)	9 (9)	8 (8)	0
Escherichia coli	10–3	0	10 (10)	9 (9)	10 (10)	8 (8)	8 ”	0
Klebsiella pneumoniae	10–4	0	10 ”	10 (10)	6 (6)	8 ”	8 ”	0
Paracolobactrum intermedium	10–1	0	8 (8)	6 (6)	8 (8)	10 ”	8 (6)	0
Proteus vulgaris	10–1	1 (1)	10 (10)	8 (8)	10 ”	10 ”	10 (10)	3 (3)
Pseudomonas aeruginosa	10–4	0	10 ”	6 (6)	8 ”	8 ”	8 (8)	0
Salmonella choleraesuis	10–1	0	9 (9)	0 †	4 (4)	6 (6)	4 (4)	0
” *typhi*	10–2	1 (1)	9 ”	8 (8)	10 (10)	10 (10)	10 (10)	1 (1)

* No. of positive post-mortem heart cultures in parentheses.

† Increasing bile salts mixture concentration to 2% resulted in mortality of 4 (0) with *N. meningitidis* and 6 (4) with *Salm. choleraesuis*.

consistently negative for the fungus except in the group that received bile salts. Four of the 8 dead mice in this group yielded positive cardiac cultures.

Since Tween 80's failure in this regard might conceivably have been due to its possible bactericidal action against the injected microorganism, 0.25 ml of the dilution of each strain used in *in vivo* tests was treated with 0.5 ml of 1% Tween 80 in test tubes for 6 hours at 37°C in shaker water bath oscillating at approximately 120/minute and then subcultured upon appropriate solid media. With exception of the meningococcus strain, all proved viable after such exposure, so that Tween 80's ineffectiveness cannot be attributed to a sterilizing effect upon the injected organisms, especially since the number of colonies obtained with the same cultures treated in the same way with physiological saline was roughly equal to that observed with Tween 80.

Hydrogen ion concentration, relative viscosity and surface tension of Tween 80 were compared with that of effective adjuvants and with saline solution to ascertain whether any physicochemical basis could be established for the former's inability to enhance mouse virulence (Table II). pH of Tween 80 solution

TABLE II. Physicochemical Characteristics of Various Adjuvants.

Adjuvant	Reaction (pH)	Relative viscosity	Surface tension (dynes/cm at 25°C)
Saline	6.05	1	76.4
Gastric mucin 5%	7.2	1.5	52.5
Bile salts mixture 1%	7.1	1	41.6
Aerosol MA .75%	6.0	1	31.4
Naccosol A 1%	6.4	1	39.0
Triton X-100 .5%	6.4	1	29.9
Tween 80 1%	5.8	1	42.0

was slightly but not significantly lower than that of the effective surfactants. With exception of 5% gastric mucin solution, whose relative viscosity was 1.5, the rest, including Tween 80, were all approximately 1, thereby confirming McLeod's observation(5) that pathogenizing activity and viscosity of adjuvants are not correlated. As for Tween 80's surface tension lowering capability, its action in this regard corresponds closely to that of bile salts mixture and is significantly greater

than mucin, both of which were highly effective in enhancing microbial virulence for mice.

Discussion. The mode of action by which hog gastric mucin exerts its action in enhancing bacterial virulence has not as yet been fully delineated(3). Among reasons ascribed for failure of many strains to produce bacteremia and fatal outcome, when injected intraperitoneally without mucin, is elicitation by their presence of a strong protective peritoneal leukocytic response which tends to destroy and localize the invading organisms thereby preventing dissemination into the blood stream. In addition, peritoneal fluid possesses bactericidal properties for certain species of microorganisms(6).

Although surface tension lowering capacity *per se* is probably not the sole determinant of an adjuvant's virulence enhancement capability as evidenced by Tween 80's failure, all agents tested that proved successful in this regard did possess this property. It is conceivable that they could interfere with contact between peritoneal phagocytes and bacteria by altering surface tension so that the former would no longer be able to envelop, engulf and destroy the latter. In addition, they may very well increase permeability of the peritoneal barrier between abdominal cavity and blood by virtue of the same property thereby permitting injected organisms to escape promptly into the blood stream and thus circumvent the protective effect of peritoneal phagocytosis and exposure to bactericidal action of peritoneal fluid.

Use of surfactants in place of gastric mucin for enhancement of microbial virulence, *e.g.*, in chemotherapeutic assays, offers a number of advantages. The former are easier to prepare, the problem of batch variation does not present itself with them and their chemical compositions are clearly defined, as is not the case with mucin. Furthermore, smaller gauge needles could be employed since they are less viscous and loss of intraperitoneally injected test material through needle puncture would be minimized.

Summary. Intraperitoneal injection of bile salts and certain surfactants into mice followed by adjusted, non-lethal inocula of a variety of different microbes results in significant enhancement of latter's virulence for mice. With few exceptions, mortality induced with these adjuvants is associated with high incidence of bacteremia involving intraperitoneally injected microorganisms.

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